Abstracts of papers presented at the

SALK-sponsored WORKSHOP

Ubiquitin and Ubiquitin-like Modifications in Viral Infection and Immunity

AUGUST 28 – AUGUST 30, 2007

NIH Natcher Conference Center
Bethesda, Maryland

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Peter Howley *Department of Pathology, Harvard Medical School, Boston, MA*

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Department of Pathology, Harvard Medical School, Boston, MA
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Department of Pathology, Harvard Medical School, Boston, MA
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Sungkyunkwan University School of Medicine, Suwon, Korea
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University of Chicago, Chicago, IL
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Georgetown University Medical Center, Washington, DC
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Lakshmi Balagopalan, Valarie A. Barr, and Lawrence E. Samelson — P5
LCMB, NCI, NIH, Bethesda, MD
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Columbia University Medical Center, College of Physicians & Surgeons, New York, NY
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Naina Barretto, Keith Wilkinson, and Susan C. Baker — P7
Department of Micro/Immuno, Loyola University, Chicago, IL
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Brianne A. Wilton, Manabu Furukawa, Yue Xiong, Klaus Fruh, and Michele Barry — P8
University of Alberta, Edmonton, Alberta, Canada
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Penn State College of Medicine, Hershey, PA
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Microbiotix, Inc., Worcester, MA
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Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
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Brookhaven National Laboratory, Upton, NY
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Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH
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School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA
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The Center for the Study of Hepatitis C, The Rockefeller University, New York, NY
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SPEAKER ABSTRACTS

Signaling through the Ubiquitin Proteasome Pathway

Jianping Jin[1], Mathew Sowa[1], Eric Bennett[1], Lulu Ang[1], Frank Stegmeier[2], Jing Chen[1], Steve Gygi[3], Stephen Elledge[2], and J. Wade Harper[1]
[1] Department of Pathology,[2] Department of Genetics,[3] and Department of Cell Biology, Harvard Medical School, Boston, MA 02115

Our work has focused on understanding the molecular make-up and biological roles of components of the ubiqutiin-proteasome system. One aspect of this work involves the identification of specificity factors for cullin-based ubiquitin ligases. We previously identified F-box proteins as ubiquitinated substrate receptors. More recently, we have identified BTB proteins as specificity factors for Cul3 and DCAF proteins as specificity factors for Cul4. These complexes are involved in diverse biological functions. In an attempt to understand the role of the ubiquitin from a global perspective, we have developed focused shRNA libraries targeting ~900 genes that compose the ubiquitin-proteasome system. Using this libray, we have screened for genes involved in the spindle checkpoint and have identified several new components of this mitotic surveillance pathway, including the Usp44 deubiquitnating enzyme, which controls the activation of the anaphase promoting complex (APC) by removing ubiquitin from the APC activator Cdc20. In new work, we have identified a new component of the machinery required for charging E2 conjugating enzymes with ubiquitin. We identified human Uba6 as a novel enzyme with sequence homology with human Ube1, the even thought to be exclusively required for activation of ubiquitin and transfer of ubiquitin to E2s. Through a series of biochemical and genetic experiments, we have found that Uba6 represents a second activating enzyme for ubiquitin which is responsible for charging a cohort of E2s, one of which (Use1) is not charged by Ube1. This data suggests the existence of an unanticipated second pathway for activation of ubiquitin and charging of E2s. This new E1-E2 pair is found throughout vertebrates and is present in the echinoderm sea urchin but is absent from anthropods, nematodes and fungi.

SUMOylation of MuLV Capsid by Ubc9 and PIASy is required for early events of infection

Andrew Yueh, Subarna Bhattacharrya, Lucia Nguyen Muntean, and Stephen P. Goff
Depts of Biochemistry and Molecular Biophysics and Microbiology, HHMI, Columbia University P & S, New York, NY 10032

Yeast two-hybrid screens carried out with the capsid protein (CA) of the Moloney murine leukemia virus as bait resulted in the identification of Ubc9 and PIASy, the E2 and E3 SUMO conjugating enzymes, as CA-interacting proteins. The binding site in CA for Ubc9 was mapped by deletion and alanine scanning mutagenesis to a consensus motif for SUMOylation at residues 202-220, and the binding site for PIASy was mapped to residues 114-176, directly centered on the Major Homology Region (MHR). Coexpression of CA and a tagged SUMO-1 protein resulted in covalent transfer of SUMO-1 to CA in vivo. Mutations of lysine residues to arginines near the Ubc9 binding site, and also mutations at the PIASy binding site, reduced or eliminated CA SUMOylation. Introduction of these mutations into the complete viral genome blocked or profoundly reduced virus replication. The mutants exhibited no defects in the late stages of viral gene expression or virion assembly. However, upon infection the mutant viruses were able to carry out reverse transcription to synthesize normal levels of linear viral DNA but were unable to produce the circular viral DNAs or integrated provirus normally found in the nucleus. Analogous experiments with the Fv1 protein as bait also resulted in the identification of Ubc9 and PIASy as interacting proteins. Tests of mutants of Fv1 for interaction and function suggested that Fv1 restriction of incoming virus required SUMOylation. Transient overexpression of PIASy, but not its family members PIAS1 or PIAS3, resulted in inhibition of MuLV-mediated transduction of reporter genomes into 293T cells. We suggest that the SUMOylation of CA by PIASy and Ubc9 is required for early events of infection and for Fv1 restriction of infection.

The HPV E6 oncoprotein - Regulator and substrate of the ubiquitin-proteasome system

Martin Scheffner
Laboratory of Biochemistry, Dept. of Biology, University of Konstanz, Germany

A prime example for the ability of viruses to redirect the ubiquitin-conjugating system for viral purposes is provided by those human papillomaviruses (HPVs) that are associated with malignant lesions of the anogenital tract. The E6 and E7 proteins of these HPVs utilize the ubiquitin-proteasome system to target a number of important negative cell regulatory proteins including p53 and pRb for degradation. E6-facilitated degradation of p53 is mediated by the cellular ubiquitin ligase E6-AP and evidence obtained in experimental cell culture systems indicates that E6/E6-AP-mediated degradation of p53 is important for the maintenance of the transformed phenotype of HPV-positive cells. However, it is also clear that E6 has oncogenic activities that are independent of p53. Numerous cellular proteins including E6BP/ERC55, IRF-3, NFX1-91, and the PDZ domain-containing proteins hDlg and hScrib have been reported to interact with E6 and some of these (NFX1-91, PDZ domain-containing proteins) are targeted by E6 for degradation. Based on the notions that the E6 proteins of HPVs that are not associated with malignant lesions ("low risk" HPVs) can be tricked into degrading hDlg and that these E6 proteins do not detectably bind to E6-AP in vitro, it was assumed that E6 targets hDlg for degradation in an E6-AP-independent manner. However, in cellulo reconstitution experiments indicate that E6-induced degradation of hDlg is mediated by E6-AP. Furthermore, low risk HPV E6 proteins can interact with E6-AP within cells indicating that the ability to interact with E6-AP is more conserved among HPV E6 proteins than previously assumed. However, the physiological relevance of the interaction of low risk HPV E6 proteins with E6-AP remains unclear. It was previously shown that binding of E6 induces auto-ubiquitination and degradation of E6-AP suggesting that E6-AP is not only utilized by E6 to target cellular proteins for degradation but also represents a target for E6. This hypothesis is supported by recent results indicating that E6-AP has growth-suppressive properties in both HPV-positive and HPV-negative cells. However, the physiological functions as well as relevant substrates of E6-AP are largely unknown. To obtain insight into the physiological functions of E6-AP and how these functions may be affected by E6, we are currently using various approaches including RNA interference-based methods combined with mass spectrometry. Finally, E6 does not only utilize the ubiquitin-proteasome system, it also appears to be a substrate of it. However, the mechanisms involved in proteasome-mediated degradation of E6 are unknown. Preliminary evidence indicates that E6-AP is not involved in E6 degradation and that E6 degradation is mediated by a yet unknown ubiquitin ligase.

Structural biology of ubiquitin-protein ligases

Ti Li, Xu Tan, and Ning Zheng
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Ubiquitin-dependent proteolysis regulates protein turnover and provides a widely used mechanism for eukaryotic cells to negatively modulate protein activities. Acting at the last step of a three-enzyme cascade (E1-E2-E3), ubiquitin-protein E3 ligases catalyze ubiquitin transfer from a ubiquitin-conjugating enzyme (E2) to the substrate and determine the specificity of the reaction. As a powerful protein destruction system, the cellular ubiquitin ligase machinery constitute a class of ideal host targets for viral exploitation. Recent studies have revealed that, upon simultaneously interacting with a cellular ubiquitin ligase machinery and a specific host protein, a number of viral proteins are capable of reprogramming the host E3 apparatus to promote ubiquitination and subsequent proteasome degradation of the host target. By eliminating proteins that are crucial for the host to combat viral infection, the viral intruders can effectively subvert the host immune surveillance using the endogenous ubiquitin-proteasome system of the infected cell. The increasing number of such viral-host interactions suggests that such a strategy is a widespread mechanism employed by a variety of pathogenic viruses. The cullin-RING ubiquitin ligase complexes represent the largest superfamily of multi-subunit E3s in eukaryotes and are frequently hijacked by viral proteins. Organized by a catalytic core consisting of a cullin scaffold protein and the RING domain protein Rbx1, the cullin-RING E3 machineries feature interchangeable substrate receptors that are anchored to the ligase platform through an adaptor. In humans, five closely related cullin proteins (Cul1 - Cul5) have been identified, each capable of assembling a distinct family of E3 complexes via a unique adaptor. I will review our recent structural studies, which have now revealed the unique and common architectures of several distinct cullin-RING E3 complexes. By determining the crystal structures of three viral-hijacked forms of cullin-RING E3s, we begin to understand the structural mechanisms underlying this emerging type of viral-host interactions. Potential targeting sites for therapeutic compounds will be discussed.

HIV and APOBEC degradation

Xiao-Fang Yu
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Baltimore, MD 21205

Human cytidine deaminases APOBEC3 (A3) proteins exhibit differential inhibitory activities against diverse endogenous retroelements and retroviruses including Vif-deficient HIV-1. In the absence of Vif, virion-packaged A3 proteins inhibit viral reverse transcription and integration. A3 also induce C-to-U mutations in the minus-strand viral DNA during reverse transcription which result in G to A hypermutations of HIV-1 genomes. HIV-1 Vif acts as a substrate receptor in a Cul5 ElonginB/C E3 ubiquitin ligase and targets the cellular antiviral proteins A3 for proteasomal degradation. We have thoroughly characterized the mechanism by which Vif functions as a substrate receptor for the Cul5-E3 ligase. He have identified a viral BC box with the consensus **(S/T)LxxxAxxx**Φ which is similar to the motif utilized by cellular Cul2 and Cul5 substrate receptors. In addition, we have characterized a novel interaction between substrate receptor and Cul5. HIV-1 Vif and other primate lentiviral Vif molecules use a zinc stabilized motif **H**x_2**YFxCF**$x_4$$\Phi$**x**$_2$**A**$\Phi$**x**$_{7\text{-}8}$**C**$x_5$**H** upstream from the BC box for Cul5 selection. Interaction of HIV-1 Vif with substrate A3 proteins maps to the N-terminal domain of Vif. Interestingly, recognition of APOBEC3G or APOBEC3F requires distinct amino acids in Vif. Further understanding of the Vif-A3 and Vif-Cul5 E3 interactions may lead to the development of novel anti-HIV therapies.

Manipulating the cellular DNA damage response by viral ubiquitin ligases

Matthew D. Weitzman
Salk Institute for Biological Studies, La Jolla, CA 92037.

The viral assault upon host cells inevitably induces antiviral responses designed to prevent completion of the virus lifecycle and spread of the infection. Overcoming the cellular responses can be achieved by modifying key cellular sensors and regulators. Among the challenges to the host cell is the viral DNA genome, which can be viewed as DNA damage. The cellular response elicited to viral DNA can either inhibit infection or be harnessed to aid aspects of the virus lifecycle. In the case of adenovirus, the linear double-stranded DNA genome is recognized by the cellular DNA repair machinery and in the absence of the E4 region genomes are joined into long concatemers that are too large to be packaged by the virus. This is accompanied by accumulation of repair factors into foci at viral replication centers, and activation of ATM and ATR signaling pathways. The wild-type adenovirus prevents the damage response and concatemer formation by inactivating the Mre11 complex, a cellular sensor of DNA damage. The viral E1b55K/E4orf6 proteins induce degradation of components of the Mre11 complex and E4orf3 can rearrange its subcellular distribution. The E1b55K/E4orf6 proteins recruit cellular Cul5 and elongins B and C to form an E3 ubiquitin ligase required for the proteasome-mediated degradation. In addition to the Mre11 complex, the E1b55K/E4orf6 proteins also ubiquitinate and degrade the p53 tumor suppressor. The E1b55K protein is important for substrate recognition and it binds to both p53 and the Mre11 complex. Studying the details of the interactions required for degradation has provided insights into how the ubiquitin pathway can be exploited to target different cellular substrates. While adenovirus dismantles the host machinery to evade processing of the viral genome, other viruses may utilize DNA repair to their own advantage. We have found that herpes simplex virus (HSV-1) infection can activate and exploit a cellular DNA damage response. Cellular DNA repair proteins accumulate at HSV-1 replication centers and aid viral replication in non-neuronal cells. The viral protein ICP0 is an E3 ubiquitin ligase that we show can prevent accumulation of cellular DNA repair factors at irradiation-induced foci (IRIF). The effect is dependent on the RING finger domain of ICP0 but proteasome-mediated degradation is not required. We propose that the ubiquitin ligase function of ICP0 prevents immobilization of DNA repair proteins at cellular sites of DNA damage, ensuring their availability to promote viral replication.

Analysis of the endoplasmic reticulum-associated degradation pathway of CD3d in live cells

Holger Lorenz, Dale Hailey, and Jennifer Lippincott-Schwartz
Cell Biology and Metabolism Branch, NICHD, NIH, Bethesda, MD 20892

Many misfolded and unassembled ER proteins are polyubiquinated and moved into the cytosol for proteasomal degradation in the process known as ER-associated degradation (ERAD). We have employed photoactivatable GFP (PA-GFP) and a dual-fluorescence labeling approach to dissect the ERAD pathway of the transmembrane T cell receptor subunit, CD3d, in living mammalian cells. CD3d-PAGFP molecules “switched on” by photoactivation exhibited a half-life of ~2h irrespective of intracellular protein expression levels. The visible molecules were highly mobile throughout the ER with no significant cytoplasmic pool, indicating that membrane sorting in the ER, rather than retrograde translocation and proteasomal degradation, is the rate-limiting step for ERAD trafficking of CD3d. Cytoplasmic destruction of CD3d was dependent on modifications of the protein achieved during membrane dislocation since CD3d-PAGFP molecules lacking a signal sequence, which were localized only in the cytoplasm, did not undergo detectable proteasomal degradation. Use of dual-labeled YFP-CD3d-□CFP to monitor retrograde translocation into the cytosol further revealed that membrane dislocation of CD3d resulted in unfolding of its cytoplasmic but not its luminal domain. These and other results provide new insights into the spatio-temporal dynamics, folding and fate of an ERAD substrate in living cells.

Visualizing TRIM5 alpha interactions with HIV-1 during restriction

Edward M. Campbell, Omar Perez, Jenny L. Anderson, and
Thomas J. Hope
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The TRIM family of proteins has recently been identified as being responsible for the cross species restriction of a number of retroviruses, including restriction of HIV-1 by the TRIM5alpha protein from rhesus monkeys. While this restriction is known to occur following the specific recognition of a capsid determinant by TRIM proteins, subsequent steps in the TRIM5 alpha mediated restriction of HIV-1 remain poorly defined, although restriction occurs early during infection, prior to the generation of late reverse transcription (RT) products. We find that proteasome inhibition relieves this TRIM5 alpha mediated block to RT accumulation, although 2 LTR circle formation and infection remains impaired, although functional preintegration complexes are formed under these conditions. By examining the fate of GFP-Vpr labeled virions in cells expressing HA tagged rhesus TRIM5 alpha, we observe that proteasome inhibition results in the accumulation of HIV-1 virions associated with rhTRIM5 alpha cytoplasmic bodies. This accumulation is specific to virions that have entered the host cell cytoplasm, as indicated by the loss of their fluorescent membrane label, and requires elements in the TRIM5 alpha SPRY domain known to be required for restriction specificity. Extended treatment with proteasome inhibitor dramatically extends the half life of GFP-Vpr labeled virions in the cytoplasm of TRIM5 alpha expressing cells. Arrest in this state results in TRIM5 alpha cytoplasmic bodies associating with ubiquitin and proteosomal subunits.
Consistent with a role for TRIM5 alpha cytoplasmic bodies during restriction of HIV-1 infection, we can observe interactions between mCherry-Vpr labeled HIV-1 virions and YFP-rhTRIM5 alpha cytoplasmic bodies during infection using live cell microscopy. We can demonstrate both the interaction of HIV-1 virions with preexisting cytoplasmic bodies as well as the de novo formation of TRIM5 alpha bodies around virions. We hope a comprehensive examination of this interaction can provide insight into the mechanism of retroviral restriction by TRIM5 related proteins.

Structural biology of ubiquitin and the proteasome

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The current understanding of structural aspects of ubiquitin's molecular interactions will be discussed, including the roles of enzymes and the many different ubiquitin-binding domains. Downstream consequences of ubiquitylation include targeting to the proteasome and the lysosome, both of which will be discussed in the light of recent structural data.

The biological significance of the degradation of PML by HSV-1 regulatory protein ICP0, a RING finger E3 ubiquitin ligase

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Herpes simplex virus type 1 (HSV-1) is an important human pathogen that establishes a latent state in neuronal cell nuclei after an initial epithelial infection. Periodically the virus reactivates to engage in an episode of lytic infection, enabling transmission between individuals. The balance between viral lytic and latent infection is of key clinical and evolutionary importance. Both viral and cellular factors are involved, including the viral regulatory protein ICP0 and host nuclear substructures known as PML nuclear bodies or ND10. ICP0 is a RING finger E3 ubiquitin ligase that localises to ND10 very early during HSV-1 infection, then brings about their disruption through inducing the degradation of PML and the SUMO-modified forms of Sp100, another key component of ND10. This talk will summarise recent biochemical studies on the E3 ubiquitin ligase activity of ICP0, and the interactions between HSV-1 genomes and protein components of ND10 during lytic and quiescent infection. Using shRNA technology we have demonstrated that both PML and Sp100 are involved in cell mediated repression of viral gene expression, and that depletion of both proteins significantly increases infection by ICP0-null mutant HSV-1. We propose that PML and other ND10 components are involved in a cellular repression mechanism that targets viral genomes as they enter the nucleus, and that this process is inactivated by the effects of ICP0 on ND10 constituent proteins.

Functional analyses of the Adenovirus E4-ORF3 protein

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One of the most interesting functions attributed to the adenovirus early region 4 open reading frame 3 protein (E4-ORF3) is its reorganization of PML nuclear bodies. These normally punctate structures are reorganized by E4-ORF3 into tracks that eventually surround viral replication centers. PML rearrangement is an evolutionarily conserved function of E4-ORF3, yet the precise reason for this activity has not yet been elucidated. PML, the protein responsible for nucleating PML nuclear bodies, is an interferon (IFN)-stimulated gene implicating the participation of this nuclear body in an innate antiviral response. We demonstrate that E4-ORF3 is critical to the replicative success of adenovirus during the IFN-induced antiviral state. When cells are pretreated with either alpha-IFN or gamma-IFN, a mutant virus that does not express E4-ORF3 is severely compromised for replication. This result suggests the functional significance of E4-ORF3 track formation is the inhibition of a PML-mediated, antiviral mechanism. Replication of the E4-ORF3 mutant virus can be rescued following the introduction of E4-ORF3 from evolutionarily divergent adenoviruses, suggesting a conserved function for E4-ORF3 inhibition of the IFN-induced antiviral state. Furthermore, E4-ORF3 inhibition of an IFN-induced response is unrelated to the inhibition of adenovirus replication by the Mre11-Rad50-Nbs1 DNA repair complex. We propose that the evolutionarily conserved function of the adenovirus E4-ORF3 protein is the inhibition of a host interferon response to viral infection via disruption of PML function. The PML protein is a member of a larger protein family termed Tripartite Motif (TRIM) proteins. TRIM proteins contain a tripartite domain structure in proximity to their N-termini that consists of a RING-finger domain, followed by one or two B-box domains and a C-terminal Coiled-Coil domain (collectively termed the RBCC domain). The order and spacing of these domains is evolutionarily conserved and thought to mediate protein:protein interactions and other functions. We implemented a proteomic approach to isolate cellular proteins that bind to E4-ORF3. We identified a novel interaction between E4-ORF3 and another TRIM family member, Transcriptional Intermediary Factor 1 alpha (TIF1alpha). TIF1alpha functions by recruiting coactivators and/or corepressors to modulate transcription. The interaction between E4-ORF3 and TIF1alpha was validated by coimmunoprecipitation and binding of recombinant proteins. Indirect immunofluoresence demonstrated that TIF1alpha is reorganized into track structures that contain PML upon E4-ORF3 expression. The RBCC domain of TIF1alpha is sufficient for E4-ORF3-induced rearrangement and TIF1alpha reorganization is conserved across adenovirus serotypes. The functional significance of this interaction is being investigated.

E1B-55K has SUMO1-p53 ligase activity required for maximal p53 inhibition

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The adenovirus E1B-55K protein has been optimized by natural selection over a vast number of generations to rapidly inactivate p53, a key component in cellular anti-viral defense. E1B-55K employs several interrelated mechanisms to completely inactivate p53, culminating in p53 degradation. Here we report an additional E1B-55K function that contributes to p53 inactivation: Using highly purified recombinant proteins, E1B-55K stimulates the sumoylation of p53 in vitro, indicating that E1B-55K is a new class of SUMO1 E3 for p53; however, we are continuing experiments to rule out other possible explanations for the stimulation of p53 sumoylation. FRAP experiments in cells transfected with expression vectors for different PML isoforms indicate that the association of E1B-55K with p53 tethers p53 in promyelocytic leukemia (PML) nuclear bodies through interactions with PML isoform IV. p53-sumoylation in PML-nuclear bodies is mechanistically linked to p53 degradation by promoting rapid exportin1-mediated nuclear export of complexes containing p53 and E1B-55K followed by dynein motor-dependent transport on microtubules to the centrosomal microtubule organizing center (MTOC) for rapid ubiquitin-mediated proteolysis. Since viruses exploit normal cellular processes, these results predict that uninfected cells use similar mechanisms to inhibit nuclear proteins via sumoylation in PML-nuclear bodies followed by export to the MTOC and rapid proteosomal degradation.

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Gained in translation: Generating viral and self MHC Class I peptide ligands from DriPs

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MHC class I molecules function to display peptides on the cell surface to enable the immune system to monitor gene expression and respond to the presence to viruses and other intracellular pathogens and viruses. There is increasing evidence that peptides predominatly derive from defective ribosomal produces (DriPs), rapidly degraded forms of otherwise stable gene products. Pulse chase experiments suggest that 20-30% of newly synthesized proteins are degraded with a half-life of less than 10 min. Approximately 25% of these rapidly degraded polypeptides (RDPs), appear to be degraded in a ubiquitin-independent manner by 20S proteasomes without the involvement of 19S regulators. This subset of RDPs, is resistant to the effects of HSC70 manipulation and is insoluble in mild detergent, and may therefore represent the most severely misfolded nascent proteins. It might be the major souce of MHC class I peptides. The nature of defects that result in rapid degradation remain to be established, but I will present recent evidence that suggest a role for misacylation of tRNA.

Cystein-ubiquitination by E3 ligases

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Kaposi's Sarcoma-associated Herpesvirus encodes two homologous E3 ligases, MIR1 and MIR2, that mediate the downregulation of several cell surface proteins thus modulating the surface architecture of infected cells. Among the proteins removed from the cell surface by MIR1 and 2 are the MHC-I molecules that play a critical role in immune surveillance. Previous studies have shown that MIR1 and 2 transfer ubiquitin to the sidechain of lysine residues in the intracytoplasmic tail of MHC-I and other substrates. In addition to this common pathway that is shared by all E3 ligases, we have previously shown that MIR1 has the unique ability of transferring ubiquitin onto MHC-I molecules lacking available lysine residues in a cysteine-dependent manner. Here we report that MIR1 and MIR2 mediated–ubiquitination is restricted by the position of the lysine residue in MHC-I intracytoplasmic domain. MIR1 prefers residues away from the transmembrane, whereas MIR2 preferentially ubiquitinates residues close to the transmembrane. By creating MHC-I mutants encoding a cysteine residue at the prefered position for MIR2, we observed that these molecules were ubiquitinated and endocytosed when coexpressed with MIR2. Together these results indicate that cysteine ubiquitination is not a property of MIR1 but is shared by MIR2 as well a probably several other E3-ubiquitin ligases.

Modulation of the ubiquitin-proteasome system in Epstein-Barr Virus infected cells

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The ubiquitin/proteasome system (UPS) regulates the activity and degradation of proteins that control vital processes such as cell cycle progression, signal transduction, differentiation and apoptosis and participates in the production of peptides for MHC class I restricted antigen presentation. Epstein-Barr virus (EBV) has evolved multiple strategies in order to manipulate this system to favor virus persistence and transformation. Inhibition of B cell receptor (BCR) signaling by inactivation of BCR-associated tyrosine kinases via LMP2-dependent capturing of UPS allows virus persistence by preventing the reactivation of the productive virus cycle. The EBV nuclear antigen EBNA1 may participate in p53 inactivation by sequestering the HAUSP/USP7 deubiquitinating enzyme (DUB). EBNA1 contains an internal glycine-alanine repeat (GAr) that prevents in cis the proteasomal degradation of EBNA1 as well as reference substrates. The GAr does not interfere with ubiquitination but affects the interaction of ubiquitinated substrates with the proteasome. Ubiquitin-dependent proteolysis is impaired in EBV carrying Burkitt's lymphoma (BL) and this correlates with resistance to apoptosis and failure to accumulate ubiquitin-conjugates in response to otherwise toxic doses of inhibitors of the proteasome. DUBs and the cytosolic subtilisin-like protease tripeptidylpeptidase II (TPPII) are upregulated in BLs and rapidly induced by overexpression of *c-myc* in normal B cells carrying estrogen-driven recombinant EBV. This demonstrates a regulatory link between *c-myc* activation and changes in proteolysis that may affect malignant transformation. RNAi interference experiments suggest that the deubiquitinating enzyme UCH-L1 contribute to the malignant phenotype of BL cells by regulating cell proliferation, adhesion and apoptosis.

Ubiquitin signaling in immunity

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The NF-κB family of transcription factors plays a pivotal role in orchestrating immune and inflammatory responses against microbial pathogens. Microbial infection triggers the activation of the IκB kinase (IKK) complex, which phosphorylates the NF-κB inhibitor IκB. The phosphorylated IκB is ubiquitinated and degraded by the proteasome, allowing NF-κB to enter the nucleus to regulate the expression of a large battery of genes involved in immune and inflammatory responses.

TRAF6 is a key signaling protein that controls IKK activation in response to microbial infection. We have previously shown that TRAF6 is a ubiquitin ligase that stimulates K63 polyubiquitination by the Ub-conjugating enzyme complex Ubc13/Uev1A. This K63-polyubiquitination activates the TAK1 kinase complex through the binding between the polyubiquitin chains and the TAK1-associated proteins, TAB2 and TAB3. TAK1 then phosphorylates and activates the IKK complex, leading to the activation of NF-κB.

We have found that K63 polyubiquitination plays a pervasive role in the activation of TAK1 and IKK in multiple signal transduction pathways, including those emanating from IL-1 receptors, Toll-like receptors, TNF receptors, and T cell receptors. Recent progress in understanding the mechanism and generality of ubiquitin-mediated activation of protein kinases in the immune response pathways will be presented.

Role of ISG15 during viral infection

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Type I Interferons (IFN) exert anti-viral and immunomodulatory effects through the induction of interferon stimulated genes (ISGs). Interferon stimulated gene 15 (ISG15) is an ubiquitin-like molecule that conjugates to over a hundred target proteins by utilizing a series of interferon induced enzymes which include the E1 (UBE1L), E2 (UbcM8), and several E3's (Herc5, EFP, HHARI). ISG15 is also released from cells following treatment with IFN and has been found in the serum of IFN treated patients, suggesting it may function as a cytokine. We have previously shown that ISG15 functions as a critical host antiviral molecule. Overexpression of ISG15 by a recombinant Sindbis virus protected IFNAR-/- mice from lethality. Futhermore, mice deficient in ISG15 displayed increased susceptibility to multiple pathogens including influenza A and B viruses, herpes simplex virus, and Sindbis virus. During the course of viral infection both ISG15 conjugates in infected tissues and free ISG15 in the sera of infected mice were detected. The mechanism by which ISG15 exerts this anti-viral activity is currently under investigation. To evaluate the role that protein conjugation plays in the antiviral activities of ISG15, we have performed *in vivo* structure-function analysis of ISG15 during Sindbis virus infection, and have evaluated the responses of mice lacking UBE1L, the ISG15 E1, to viral infection. Results from these studies will be discussed.

ISG15 and ISG15 deconjugating enzyme UBP43 (USP18) in innate antiviral responses

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Interferons (IFNs) regulate diverse immune functions through the transcriptional activation of hundreds of genes involved in anti-viral responses. IFN inducible ubiquitin-like protein ISG15 and ISG15 deconjugating enzyme UBP43 (USP18) are highly expressed in cells in response to a variety of stress conditions, such as viral or bacterial infection. ISG15 is present in its free form or conjugates to other cellular proteins upon IFN stimulation. UBP43 deficient cells accumulate much more ISGylated proteins and are hypersensitive to IFN treatment to enhance their antiviral responses. Our studies have demonstrated that the hypersensitivity to IFN is independent of protein ISGylation and that UBP43 is a novel inhibitor of Type I IFN signaling which blocks the interaction between IFN receptor subunit IFNAR2 and JAK1. Most recently, we have revealed that ISG15 inhibits the egress of rhabdo- and filoviruses from the infected cells and therefore enhances the innate anti-viral response. Nedd4 Ub E3 ligase, an integral component of viral budding machinery, is negatively regulated by ISG15 through direct protein-protein interaction. Overexpression of ISG15 diminishes the ability of Nedd4 to ubiquitinate viral matrix proteins, which in turn leads to a decrease in budding of VSV and Ebola VP40 virus-like particles from the cells. Free ISG15 specifically binds to Nedd4 and blocks its interaction with Ub-E2 molecules thus preventing further ubiquitin transfer from E2 to E3 molecules. These results implicate ISG15 as a critical player in the IFN-mediated inhibition of viral budding and point to a mechanistically novel function of ISG15 in the enhancement of the innate antiviral response through specific inhibition of Nedd4 ubiquitin E3 activity.

Endosomal quality control of CD4-lck assembly state mediated by ubiquitination-dependent sorting to the Multivesicular Body pathway

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CD4 is a type I transmembrane glycoprotein that is expressed on a subset of T lymphocytes and macrophages/monocytes as a complex with the tyrosine kinase lck. CD4 binds MHC class II molecules on antigen-presenting cells and transduces activating signals via lck. In addition, CD4 serves as a co-receptor for HIV and is a target for downregulation by the HIV Nef protein. Previous studies showed that association with lck decreased the internalization and degradation of CD4. To investigate the mechanism involved, we examined the determinants of CD4 internalization and degradation, and their regulation by assembly with lck. We found that CD4 expressed in the absence of lck in HeLa cells was transported to the plasma membrane, upon which it became ubiquitinated on four lysine residues within its cytosolic tail. Mutation of these four lysine residues did not affect the rate of CD4 internalization but increased its half-life due to decreased targeting for degradation in lysosomes. Interference with the multivesicular body (MVB) pathway by either RNAi or overexpression of some of its components inhibited CD4 degradation and resulted in accumulation of CD4 in aberrant endosomal intermediates, thus demonstrating that CD4 follows the MVB pathway to lysosomes. Depletion of the ubiquitin ligase Nedd4 by RNAi caused a dramatic reduction of CD4 ubiquitination and lysosomal degradation. Co-precipitation and yeast two-hybrid analyses showed binding of Nedd4 to the CD4 cytosolic tail. Expression of lck decreased Nedd4 binding, ubiquitination and MVB/lysosomal targeting of CD4. Expression of the HIV Nef protein, on the other hand, had no effect on CD4 ubiquitination, though it increased its internalization and MVB/lysosomal targeting through the action of dileucine-based sorting signals in both CD4 and Nef. These observations indicate that assembly with lck controls the fate of cell surface CD4 by regulating its ubiquitination-dependent targeting to the MVB/lysosomal pathway. This can be viewed as an example of endosomal quality control whereby CD4-lck complexes are stably expressed at the cell surface while unassembled CD4 is targeted for disposal.

The Herpesvirus ubiquitin-specific proteases

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All members of the herpesvirus family encode in their large tegument protein an enzymatic activity that catalyzes the hydrolysis of ubiquitin isopeptide bonds. We discovered this activity by using an activity based probe, composed of an epitope tagged version of ubiquitin, onto which was installed an electrophilic function to target cysteine proteases involved in the reversal of ubiquitin modification. Bioinformatic strategies failed to detect this activity, and the assignment of an enzymatic property to the large tegument protein was therefore unexpected. This ubiquitin-specific protease activity can be detected in smaller subdomains of the tegument protein, and requires the approximately 250 N-terminal residues of the tegument protein for robust activity. We have characterized its occurrence in cells infected with alpha, beta or gamma herpesviruses, and find evidence for its presence in all species examined to date. We have solved the structure of the MCMV ubiquitin specific protease embedded in the M48 protein in a complex with the ubiquitin based electrophilic probe. Our analysis shows that the protease assumes an overall fold that resembles that of the papain catalytic core, but the disposition of the active site residues is clearly distinct from that of other ubiquitin-specific proteases. In a collaborative effort with Dr K. Osterrieder and his colleagues at Cornell, we have analyzed also the properties of the ubiquitin specific protease encoded by Marek's disease virus. A single point mutant made in Marek's disease virus to abolish the ubiquitin-specific protease activity large eliminates the ability of the virus to cause T cell lymphomas, whilst the effects of this mutation on virus growth appear modest, as has been seen also for corresponding mutants for the other herpesviruses. Although the important task of identifying the true substrate(s) of this enzyme and its role in the virus life cycle are not yet known, we may conclude that the herpesvirus ubiquitin-specific proteases are likely to have a major impact on the replicative success of these viruses in vivo. Accordingly, this activity is an attractive target for small molecule based strategies. Our results also have implications for the design of attenuation strategies that might be helpful for vaccine production.

Structure and functional studies of the papain-like protease of SARS coronavirus: a viral deubiquitinating enzyme and interferon antagonist

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Coronavirus (CoV) infection of humans can result in diseases ranging from common colds to severe acute respiratory syndrome (SARS) with 10% mortality. To date, no vaccines or antiviral drugs are available to treat any coronavirus infection. For SARS-CoV, two distinct proteases, a chymotrypsin-like protease (3CLpro) and a papain-like protease (PLpro) are required to process the replicase polyprotein, and these proteases are attractive targets for antiviral drug development. We found that PLpro processes the replicase polyprotein at three sites to release nonstructural proteins 1, 2 and 3. Interestingly, the cleavage sites recognized by PLpro are similar to those recognized by deubiquitinating (DUB) enzymes. To gain further insight, we solved the structure of SARS-CoV PLpro and characterized the enzymatic activity. The structure of PLpro revealed the catalytic triad (Cys-His-Asp), zinc-binding domain, the ubiquitin-like N-terminal domain and overall structural similarity to known deubiquitinating enzymes USP14 and HAUSP. We found that PLpro recognizes LXGG processing sites and exhibits deubiquitinating activity with a preference for K-48 linked polyubiquitin. Analysis of papain-like proteases from murine coronavirus MHV-A59 and human CoV-NL63 revealed that DUB activity is conserved in coronaviruses. In addition, we found that SARS-CoV PLpro is a potent interferon (IFN) antagonist. PLpro is involved in an interaction with IRF-3, inhibits phosphorylation and nuclear translocation of IRF-3, which disrupts the activation of type I IFN responses. PLpro IFN antagonism is independent of protease/DUB activity. Studies are currently underway to determine the role of DUB activity in coronavirus replication and pathogenesis and to develop antivirals that inhibit coronavirus papain-like protease and IFN antagonism activities.

ESCRTs in membrane trafficking at the endosome

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The endosomal membrane trafficking system is required for many key cellular processes including down-regulation of activated cell-surface receptors, antigen presentation, and the sorting of biosynthetic cargoes to the lysosome. The Endosomal Sorting Complex Required for Transport (ESCRT) is essential for the sorting of transmembrane proteins/receptors into Multi-Vesicular Bodies (MVB) and into lysosomes. Thus the ESCRT complexes contribute critically to the regulation of cell surface receptor signaling during development and disease. Monoubiquitination of both biosynthetic and endocytic cargo by the Rsp5 HECT-domain Ub ligase in yeast serves as a critical signal for sorting into late endosomal multivesicular compartments. The yeast Vps27 protein and its mammalian homolog HRS are required for the formation of MVBs. We have identified three distinct protein ESCRT complexes I, II and III that function in the recognition and sorting of ubiquitinated MVB cargoes. Vps27 binds to both Ub (cargo) and the phosphoinositide PI3P on the surface of the endosome where it functions as a docking site for the ESCRT-I complex, thereby initiating the MVB sorting reaction. ESCRT-I recruits/activates the ESCRT-II complex. Together with Roger Williams' lab, we have characterized the role for the GLUE domain in ESCRT-II, its lipid (PI3P) binding and its interaction with ESCRT-I. Our observations indicate that both PI3P and monoubiquitin function as critical signals for the selective recruitment and activation of the endosomal sorting machinery required for receptor down-regulation. The mechanisms of assembly and funcion as well as the regulation of the ESCRT machinery are still poorly understood. Recently, we identified Mvb12 (multivesicular body sorting factor of 12 kDa) as a novel component of the ESCRT-I complex. Our data suggests that Mvb12, the first known regulator of the ESCRT complexes, maintains ESCRT-I in an inactive state in the cytosol and regulates the assembly of ESCRT-I and -II on the endosome, an essential step for cargo sorting in the MVB pathway. We have begun to address the molecular mechanisms that drive assembly and dynamics of the ESCRT-III lattice. ESCRT-III function is required for the final protein-sorting step and membrane invagination during MVB vesicle formation. Our results strongly indicate that the assembly of ESCRT-III on endosomes requires an activating conformational switch from an inactive cytoplasmic monomer. This activation is mediated by ESCRT-II. Once activated, ESCRT-III subunits assemble on endosomal membranes in a tightly regulated cascade-like manner. Based on our results, we propose that that different subunits of ESCRT-III, although biochemically and structurally very similar, perform very specific functions during the assembly of ESCRT-III on endosomes to regulate the final steps in the MVB sorting process.

Ubiquitin in HIV budding

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The HIV Gag protein coordinates viral trafficking, membrane binding, assembly, cofactor packaging, budding, and maturation. Late in the infectious cycle, Gag assembles on plasma and on endosomal membranes where it forms enveloped particles that bud through the membrane. Previous work has shown that efficient HIV release requires both the covalent transfer of ubiquitin and the actions of two cellular proteins, TSG101 and AIP1, which bind directly to conserved peptide motifs within the p6 domain of Gag. TSG101 and AIP1 normally function in the process of vesicle formation at late endosomal compartments called multivesicular bodies (MVB). Thus, it appears that HIV budding and MVB vesicle formation are analogous processes. Our lab, and others, have been investigating the biochemistry of HIV release and MVB biogenesis, and have now identified some 27 human proteins that function in these pathways. I will discuss the structures and functions of protein complexes in the pathway, with particular emphasis on the roles of ubiquitin in HIV release and MVB budding. Specifically, I will discuss how two of the multiprotein ESCRT (Endosomal Sorting Complexes Required for Transport) complexes bind ubiquitin and how ubiquitin ligases may function in the pathway.

Pathway discovery in the ubiquitin system: Application of RNAi and proteomic technologies

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The ubiquitin-proteasome system is involved in virtually every biological process that occurs in cells and is the target for viral subversion. Analysis of the human genome suggests that it encodes more than 950 genes that are linked in one way or another to the process of conjugation or removal of ubiquitin or ubiquitin-like proteins to other proteins. The majority of these proteins are unstudied. Our recent work seeks to develop approaches and technologies that will allow the role of the ubiquitin system to be interrogated in diverse biological systems. The two core technologies include RNAi libraries and proteomic approaches that facilitate the identification of signaling pathways and targets of the ubiquitin machinery. In this workshop, we will describe the development of shRNA libraries targeting the ubiquitin system and the use of these libraries to identify genes involved in cell cycle and checkpoint control, including shRNA vector design, assay development and target validation. Understanding the complexity of the ubiquitin pathway requires approaches that define the molecular contexts in which particular components of the system function. Efforts in the development of semi-highthroughput proteomics for the identification of protein complexes involved in the ubiquitin pathway will be discussed, including the development of software that facilitates analysis and integration of proteomic data. Examples of how proteomic and RNAi approaches can be integrated will be discussed.

Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway

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The ubiquitin-proteasome system (UPS) comprises hundreds of different conjugation/deconjugation enzymes and multiple receptors that recognize ubiquitylated proteins. A formidable challenge to deciphering the biology of ubiquitin is to map the networks of substrates and ligands for components of the UPS. Several different receptors guide ubiquitylated substrates to the proteasome, and neither the basis for specificity nor the relative contribution of each pathway is known. To address how broad of a role the ubiquitin receptor Rpn10 (S5a) plays in turnover of proteasome substrates, we implemented a method to perform quantitative analysis of ubiquitin conjugates affinity-purified from experimentally-perturbed and reference cultures of *Saccharomyces cerevisiae* that were differentially labeled with ^{14}N and ^{15}N isotopes. Shotgun mass spectrometry coupled with stable isotope ratiometric quantification and statistical analysis based on q-values revealed ubiquitylated proteins that increased or decreased in level in response to a particular treatment. We first identified over 225 candidate UPS substrates that accumulated as ubiquitin conjugates upon proteasome inhibition. To determine which of these proteins were influenced by Rpn10, we evaluated the ubiquitin conjugate proteomes in cells lacking either the entire Rpn10 (*rpn10*Δ) or only its UIM poly-ubiquitin binding domain (*uim*Δ). Twenty seven percent of the UPS substrates accumulated as ubiquitylated species in *rpn10*Δ cells, whereas only one-fifth as many accumulated in *uim*Δ cells. These findings underscore a broad role for Rpn10 in turnover of ubiquitylated substrates, but a relatively modest role for its ubiquitin-binding UIM domain. This approach illustrates the feasibility of systems-level quantitative analysis to map enzyme-substrate networks in the UPS.

POSTER ABSTRACTS

Induction of PML desumoylation by the human cytomegalovirus IE1 protein: a role in viral growth and the possible mechanisms

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In human cytomegalovirus (HCMV)-infected cells, the immediate-early IE1 protein disrupts the subnuclear structures known as the PML oncogenic domains (PODs) or nuclear domain 10 (ND10), via the induction of PML desumoylation. The central hydrophobic domain of IE1 is required for both PML binding and induction of PML desumoylation and this activity correlates with the functions of IE1 in transcriptional regulation in reporter assays. To address the role of IE1-induced PML desumoylation in viral growth, a mutant HCMV encoding IE1(D290-320) that was defective in this activity was generated. This mutant virus displayed severe growth defects compared to wild-type and its revertant viruses, suggesting that IE1-induced PML desumoylation is an important event in productive viral infection. In in vitro assays to study the mechanism by which IE1 uses to induce PML desumoylation, IE1 exhibited neither inhibitory effect on sumoylation of PML nor intrinsic SUMO protease activity against PML. Although both IE1 and SUMO proteases efficiently desumoylated PML in cotransfection assays, they exerted different effects on the localization of PML. In cells transfected with SUMO proteases, the number of PML foci was reduced significantly and these remnant PML foci were devoid of SUMO-1 signals. However, in cells cotransfected with both SUMO proteases and IE1, these SUMO-independent PML foci were also completely disrupted. Furthermore, IE1, but not SENP1, was shown to disrupt the PML foci generated via transfection of a sumoylation-deficient mutant of PML. Therefore, inhibition of PML oligomerization by IE1 appears to play an important role in the induction of PML desumoylation in HCMV-infected cells.

Nef promotes HIV-1 evasion of a proteasome-dependent postentry restriction

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The HIV-1 accessory protein Nef promotes HIV-1 infection by an undefined mechanism. Nef facilitates an early post-entry step in the virus life cycle, yet no molecular or structural defect has been specifically linked to the impaired infectivity of Nef-defective HIV-1 particles. We show that expression of Nef reduces the level of ubiquitylated proteins in virions. Genetic inhibition of protein ubiquitylation in virus-producing cells preferentially enhanced the infectivity of the resulting Nef-defective HIV-1 particles. Additionally, viral cores purified from Nef-defective HIV-1 particles exhibited elevated sensitivity to proteasomal degradation *in vitro*. Addition of proteasome inhibitors rendered target cells fully permissive to infection by Nef-defective HIV-1 particles. Collectively, these results suggest that Nef enhances HIV-1 infection by limiting ubiquitylation of virion-associated proteins, thereby promoting escape from proteasome-dependent restriction of the viral core in the target cell. Collectively, our results reveal a novel mechanism for viral evasion of a proteasome-dependent antiviral activity.
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Human papillomavirus E6 protein activates NF-κB by inhibiting CYLD-mediated loss of TRAF2

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Human papillomavirus (HPV) genital infections are highly prevalent, with 20 million Americans currently infected, and 5.5 million new cases annually. Low-risk HPV types cause condylomata and low-grade anogenital dysplasias; high-risk HPV types cause condylomata and low-grade dysplasias, but also underlie high-grade dysplasias and anogenital malignancies. Differences in the activity of the high- and low-risk E6 oncoproteins may underlie differences in the oncogenic potential between high and low-risk HPV types. High risk, but not low-risk E6 proteins promote proteasomal degradation of p53.

In an effort to understand what other roles high- and low-risk E6 proteins may play in promoting growth and malignant transformation of epithelial cells, we have demonstrated that HPV E6 activates NF-κB expression, and that this activation may be mediated through E6 interaction with the cylindromatosis tumor suppressor protein (CYLD), a ubiquitin protease and known suppressor of NF-κB activation. Using a dual luciferase reporter assay system, we have found that expression of HPV16 E6 in mammalian cells reverses CYLD-mediated suppression of NF-κB pathway activation. We have also found that both high- and low-risk E6 proteins can be co-precipitated with CYLD. Further studies indicate that CYLD expression in mammalian cells leads to loss of TRAF2. This CYLD-mediated loss of TRAF2 is potently inhibited by high-, but not low-risk E6 proteins. We are now working to assess the role of E6:CYLD interaction in HPV pathogenesis.

RAG1 RING finger domain substitutions reduce V(D)J recombination in Pro-B cells

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RAG1 is part of the recombinase that carries out V(D)J recombination, a programmed DNA rearrangement required for antigen receptor assembly in developing lymphocytes. Certain missense mutations throughout the RAG1 protein coding region have been associated with Omenn syndrome (OS), a rare primary immunodeficiency characterized by the absence of circulating B cells and T cell oligoclonality. Most mutations associated with OS are found in the region of RAG1 minimally required for DNA cleavage (the RAG1 “core”); however, the G1095A mutation, resulting in a C328Y amino acid substitution, disrupts a non-core region of the protein recently demonstrated to possess ubiquitin ligase activity. We studied the affects of the equivalent substitution (C325Y) in the mouse RAG1 protein (mRAG1). mRAG1 C325Y demonstrated a severe defect in recombination of extra-chromosomal substrates in cultured murine pro-B cells. Both types of DNA joints normally formed during V(D)J recombination were severely reduced relative to the wild type protein, with signal joints being reduced 50-100 fold and coding joints being undetectable. In agreement with this, mRAG1 C325Y also showed a reduced ability to carry out D to J recombination on the murine IgH locus. An adjacent substitution, P326G, also showed a reduced ability to support plasmid recombination, but unlike C325Y, the recombination defect in P326G could be partially masked by high levels of protein expression. The severe defect in mRAG1 C325Y could not be explained by reduced protein expression or inappropriate intra-cellular distribution. Other work in our laboratory demonstrates that both C325Y and P326G abrogate RAG1 ubiquitin ligase activity. Substitutions in this domain that retain ubiquitin ligase activity supported recombination at levels comparable with wild type. Thus there was a correlation between the ability to support ubiquitin ligase activity and the ability to support recombination. These data indicate the importance of an intact RING finger to support optimum levels of V(D)J recombination and suggest a role for RAG1 ubiquitin ligase activity in normal B cell development.

Cbl-dependent regulation of LAT-nucleated signaling complexes

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T cell receptor (TCR) activation results in the formation of multiple signaling complexes and stimulation of intracellular signaling pathways vital for proper immunological function. We have studied the trafficking of a number of signaling proteins in activated T cells. Upon receptor activation, the adapters LAT and SLP-76, visualized as chimeric proteins tagged with YFP, transiently associate and then rapidly dissociate from the TCR, following which clusters containing LAT and SLP-76 dissipate. The migration away from the initial signaling clusters and dissipation of LAT and SLP-76 could reflect mechanisms that attenuate the immune response of the T cell. We demonstrated in a previous study that SLP-76 is endocytosed via a clathrin-independent, lipid raft-dependent pathway involving ubiquitination. Consequently, we investigated whether the E3 ubiquitin ligase c-Cbl might control the fate of these molecules. Fluorescently tagged Cbl transiently co-localizes with LAT and SLP-76 clusters at the start of T cell activation, followed by dissipation of Cbl clusters. In contrast, internalization of LAT and SLP-76 was severely inhibited in cells expressing versions of the ubiquitin ligase c-Cbl mutated in the RING domain, which mediates ubiquitin ligase activity. Inhibition of LAT internalization by RING domain mutants required the TKB and proline-rich domains of c-Cbl. Moreover, c-Cbl RING mutants suppressed LAT ubiquitylation and caused an increase in cellular LAT levels. Collectively, these data suggest that following rapid formation of signaling complexes upon TCR stimulation, c-Cbl RING domain activity is involved in the internalization and possibly downregulation of a subset of activated signaling molecules.

Modification of MDA-5 and RIG-I during poliovirus infection

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The cytoplasmic RNA helicases MDA-5 and RIG-I are components of the innate immune system that sense RNA virus infections. We found that MDA-5 protein is degraded in poliovirus-infected cells starting at 4 h post-infection. MDA-5 is not cleaved by either of the poliovirus proteinases, $2A^{pro}$ and $3C^{pro}$, but rather by cellular caspases and the proteasome. Cleavage of poly(ADP-ribose) polymerase (PARP), an indicator of early apoptosis, correlates with cleavage of MDA-5 during poliovirus infection. When apoptosis is induced with puromycin, both PARP and MDA-5 cleavage are observed. The MDA-5 cleavage product observed in cells treated with puromycin and in poliovirus-infected cells is 90 kDa. Cleavage of RIG-I also occurs during poliovirus infection, and is mediated by the viral proteinase $3C^{pro}$. Experiments are currently under way to determine if poliovirus-induced cleavage of MDA-5 and RIG-I is a mechanism to antagonize production of type I interferon in response to viral infection. Recently ubiquitinylation of RIG-I has been shown to be important to mediate its antiviral function. We found that MDA-5 is also ubiquitinylated, and we are exploring the role of this modification in cleavage of MDA-5 during poliovirus infection.
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Deubiquitinating activity of the SARS-CoV papain-like protease

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The SARS-CoV papain-like protease, PLpro, is involved in proteolytically processing the replicase polyprotein into mature, non-structural proteins, which assemble into a membrane-associated replicase complex. Previously, we and others demonstrated that a core domain of PLpro has deubiquitinating (DUB) activity in *invitro* assays (Barretto et al, 2005, Lindner et al, 2005). DUB activity and proteolytic activity depend on the same catalytic triad. Crystal structure of this core revealed similarity to cellular DUB enzymes, HAUSP and USP14, and prominent structural features such as a Zn-binding finger and an amino-terminal ubiquitin-like domain (Ratia et al, 2006). We characterized the specificity of PLpro DUB activity, against K48 and K63 polyUb chains and found that WT PLpro cleaves K48-linkages preferentially. To understand the effect of PLpro in the context of the host-cell environment, we transfected the core domain of PLpro into cells and used an activity-based probe, HA-Ub-VS, which forms an irreversible adduct with the active-site cysteine residue of DUB enzymes. This probe recognized and bound active PLpro but not the catalytic cysteine mutant. We also demonstrate that this core domain recognizes and deubiquitinates cellular substrates. In the context of coronavirus infection, PLpro is membrane- bound. To determine the effect of membrane-association on DUB activity, we added a neighboring transmembrane domain to anchor the soluble PLpro to membranes. Membrane-bound PLpro still possesses DUB activity. This shows that PLpro DUB activity can recognize and process Ub-linked targets and may modulate multiple processes in coronavirus-infected cells.
Current efforts focus on identifying binding partners of PLpro. Guided by the available crystal structure, we are investigating the role of the Ubl-domain, zinc-binding finger and solvent-exposed residues of PLpro in interactions with its substrates.

Poxvirus modulation of the host ubiquitin machinery

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Poxviruses encode numerous immunomodulatory proteins, including proteins capable of utilizing the ubiquitin protein modification pathway. We have recently shown that strains of ectromelia virus and vaccinia virus encode a unique ubiquitin ligase, p28, which localizes to the viral DNA replication factories by virtue of an N-terminal DNA binding domain. In addition, poxviruses encode numerous ankyrin repeat proteins with putative F-box domains and are the only viruses known to contain proteins with both BTB (Bric-a-Brac, Tramtrack, Broad complex) and kelch domains. Cellular proteins containing BTB/kelch domains function as adapters for the recruitment of substrates to cullin-3 based ubiquitin ligases suggesting that the poxvirus encoded BTB/kelch proteins may modulate the ubiquitin pathway through interaction with cullin-3. Ectromelia virus, the causative agent of lethal mousepox, encodes four BTB/kelch proteins and one BTB-only protein. Our studies have found that two of the ectromelia virus encoded BTB/kelch proteins, EVM150 and EVM167, interact with cullin-3. Similar to cellular BTB proteins, the BTB domain of EVM150 and EVM167 is necessary and sufficient for cullin-3 interaction. During infection EVM150 and EVM167 localize to discrete cytoplasmic regions. Significantly, poxvirus infection dramatically altered the subcellular distribution of cullin-3 resulting in the co-localization of cullin-3 with EVM150 and EVM167. Both EVM150 and EVM167 supported the formation of active cullin-3 based ubiquitin ligases as demonstrated by an in vitro ubiquitin ligase assay and EVM150 and EVM167 co-localized with conjugated ubiquitin as assessed by confocal microscopy and immunoprecipitation. Our findings suggest that the ectromelia virus encoded BTB/kelch proteins, EVM150 and EVM167, interact with cullin-3 potentially recruiting currently unidentified substrates for ubiquitination. Our observations further suggest that poxviruses have evolved multiple mechanisms to modulate the host ubiquitin machinery in order to ensure virus replication and survival.

Nucleocapsid dephosphorylation is Dispensable for duck hepatitis B virus secretion

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The dynamic phosphorylation status of the hepadnavirus nucleocapsid (NC) is known to regulate viral DNA synthesis, which takes place within the NC. In particular, NC dephosphorylation appears to be correlated with mature DNA synthesis, NC envelopment and virion secretion. To understand the functional significance of core dephosphorylation in virion secretion, we expressed the duck hepatitis B virus (DHBV) core mutants in which the phosphorylation sites were converted to aspartate to mimic constitutive phosphorylation using transient transfections, and analyzed the effect of the aspartate substitutions on virion secretion into the medium. Though the synthesis of the mature relaxed circular DNA within the NC was reduced in the mutants, a small amount of mature NC was secreted into the medium. These results thus indicated that NC dephosphorylation was dispensable for virion secretion. Further, we verified that RnaseH$^-$ mutant forming RNA-DNA hybrid too was secreted into the medium, but with less efficiency. Detailed analysis of the genome maturity in secreted virions, using both the core mutants as well as the RnaseH$^-$ mutant, indicated a double-stranded nucleic acid, whether a DNA or RNA-DNA hybrid, was able to trigger NC envelopment, provided the double-stranded molecule reached a threshold of approximately 2 kilobasepairs. We are also investigating the role of potential host factor(s), including those involved in protein ubiquitination pathway, that may facilitate the duck hepatitis B virus secretion.

Biochemical characterization of cellular receptors for ebola virus

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Ebola virus (EBOV) infection is initiated by interactions between the viral envelope glycoprotein (GP) and its cognate receptor(s). To investigate cell surface determinants important for EBOV infection, we used a human immunodeficiency virus (HIV)-based pseudotyped virus as a surrogate system. The infectivity of the pseudotypes was determined by quantitating the expression of the luciferase reporter gene present in the HIV backbone. Treatment of 293T cells with pronase and phospholipase C inhibited the pseudotype virus infectivity. In contrast, heparinase treatment had no effect on pseudotype virus infection. In another approach to characterize the EBOV receptor, we used nonionic detergent octyl-β-glucoside (OG) for non-cytolytic extraction of the 293T cell surface proteins and lipids. Preincubation of the pseudotype viruses with OG extract inhibited the virus infectivity by ~80% at ~ 50 μg of protein per ml. This inhibition was removed when the extract was either heat treated or treated with pronase, indicating the protein nature of the receptor. Treatment of cells with β-cyclodextrin reduced the infectivity of the pseudotype. The involvement of cell surface proteins, phospholipids, and cholesterol in pseudotype infection suggests that the virus receptor(s) may be a part of lipid microdomains or rafts present in the cell membrane. These rafts have been proposed to be composed of cholesterol, glycosphingolipids, and a specific set of associated proteins. They are thought to function as specialized platforms for apical cell sorting of proteins and signal transduction. The results will be verified by using infectious EBOV (Zaire strain) in the BSL4 laboratory at US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. Further experiments to identify and characterize the proteins that block EBOV infections are in progress.

The KSHV encoded K5 RING recruits multiple E2 enzymes for polyubiquitination of MHC class I

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Ubiquitination has emerged as a critical mechanism for receptor downregulation. We, and others, identified the K5 gene product of Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8) as an E3 ligase which downregulates several important immunoreceptors including class I, ICAM-1, CD1d, PECAM, and B7.2. K5 is a type III integral membrane protein with an N-terminal RING-CH domain and similar E3 ligases have been identified in other viruses. K5 polyubiquitinates class I in a post-ER compartment and we find this polyubiquitination is lysine-63 linked. The membrane proximal lysine of HLA A2 (K335) is the primary target of K5. We have identified multiple E2 conjugating enzymes which *in vivo* mono- and polyubiquitinate the MHC class I molecules.

Analysis of poly-ubiquitin chain assembly catalysed by a viral RING-finger E3 ubiquitin ligase

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The herpes simplex virus type-1 (HSV-1) regulatory protein ICP0 stimulates the onset of viral lytic replication and the reactivation of quiescent viral genomes from latency. In order for ICP0 to mediate these processes it requires its zinc-binding RING-finger domain, a structural motif that confers E3 ubiquitin ligase activity. During infection, ICP0 has been shown to target a number of cellular proteins for degradation in a proteasome-dependent manner, a process that is thought to inhibit and/or relieve cellular repression mechanisms that either instigate and/or maintain viral DNA genomes in a state of transcriptional quiescence.

Like many RING-finger ubiquitin ligases, ICP0 can efficiently induce the formation of unanchored poly-ubiquitin chains in vitro in the presence of the E1 ubiquitin activating enzyme, ubiquitin, and either of the two E2 ubiquitin conjugating enzymes, UbcH5a or UbcH6. However, little is known about how ICP0 catalyses the formation of poly-ubiquitin, and in particular what isopeptide ubiquitin linkages it utilizes during chain assembly. Using a panel of ubiquitin mutants, LC-MSMS mass spectrometry and confocal microscopy we show that ICP0 is capable of catalyzing the formation of poly-ubiquitin using multiple ubiquitin linkages, predominantly including K11, K48, and K63 linked chains, independent of its associated E2. In contrast however, microscopy analysis demonstrated that ICP0 is capable of inducing the formation of isopeptide linked ubiquitin conjugates in a manner that is not dependent on the presence of any single lysine residue within ubiquitin itself. The relevance of these results is discussed.

Mss1, a proteasome-associated ATPase, is recruited to virus replication compartments and interacts with viral proteins during HSV-1 lytic infection

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We have previously demonstrated that the 20S proteasomal core particle is sequestered within discrete nuclear foci of Herpes Simplex Virus Type 1 (HSV-1)-infected cells [1, 2]. The 19S regulatory particle enhances 20S proteasome function and the ATPase subunits of the 19S, when detached from the proteasome, have activities related to DNA repair, transcription, and translation [3]. We have found that the 19S complex is uncoupled from the 20S core proteasome and at least one of its ATPase subunits, Mss1, is recruited to viral-specific sites and associates with HSV-1 proteins involved in DNA metabolism/translation initiation. Using several techniques, we have found that Mss1 interacts with the viral ICP6 protein and this interaction is independent of RNA or DNA. We do not yet know if this interaction is direct or whether the Mss1/ICP6 complex contains additional proteins. ICP6 has recently been proposed to provide a scaffold-like function to stabilize translation-elongation complexes [4]. We are interested in whether Mss1 association facilitates or influences this activity of ICP6 as well as the other activities of Mss1 during infection. Although the use of other proteasomal ATPases have been reported for some viral systems [5, 6], to the best of our knowledge, a role for Mss1 in HSV-1 viral replication has not been previously reported. We are currently using siRNA technology to reduce the levels of Mss1 and analyzing the consequence of this on lytic infection. These studies will reveal the reliance of HSV-1 on Mss1 and provide information about this protein's overall biological functions. Ultimately, we aim to understand whether unique interactions made between viral and cellular proteins can be targeted in the development of novel, specific and effective antiviral therapies directed at the host-pathogen interface.

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E1-L2, a novel ubiquitin E1-like protein, activates both ubiquitin and FAT10

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Ubiquitination is one of many post-translational modifications in eukaryotes. Three enzymes (E1, E2, and E3) are involved in conjugating ubiquitin to protein substrates. We report here the identification of a novel E1-like protein, E1-L2, which is homologous to the ubiquitin E1 and another E1 involved in the activation of the ubiquitin-like protein ISG-15 (E1-L1). We found that E1-L2 activates both ubiquitin and another ubiquitin-like protein FAT10. Interestingly, E1-L2 can transfer ubiquitin to Ubc5 and Ubc13, but not Ubc3 and E2-25K, suggesting that E1-L2 may be specialized in a subset of ubiquitination reactions. E1-L2, but not E1 or E1-L1, forms a thioester with FAT10 in vitro. The formation of the thioester bond requires the active site cysteine residue of E1-L2 and the C-terminal diglycine motif of FAT10. Furthermore, endogenous FAT10 forms a thioester with E1-L2 in cells stimulated with tumor necrosis factor-alpha and interferon-gamma, which induce FAT10 expression. Silencing of E1-L2 expression by RNAi impaired the formation of FAT10 conjugates in cells. To study the in vivo function of E1-L2, we ablated the E1-L2 gene in mice by homologous recombination. Surprisingly, E1-L2 deficient embryos died before embryonic day 6.5, suggesting that E1-L2 is essential for early embryo development. Since the FAT10-deficient mice develop normally, we propose that specific ubiquitination reactions catalyzed by E1-L2 play an important role in animal development.

Itch-mediated ubiquitinylation of Ig-beta dictates the endocytic fate of the B cell antigen receptor

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In both infection and autoimmunity, the development of high affinity antibodies and memory requires B cells to efficiently capture and process antigens for presentation to cognate T cells. While a great deal is known about how antigens are processed, the molecular mechanisms by which the B cell receptor (BCR) captures antigen for processing are still obscure. Herein, we demonstrate that the Ig-beta component of the BCR is diubiquitinylated and that this is dependent on the E3 ligase Itch. Itch$^{-/-}$ B lymphocytes manifest both a defect in ligand-induced BCR internalization and endocytic trafficking to late endosomal antigen processing compartments. In contrast, analysis of ubiquitinylation-defective receptors demonstrated that the attachment of ubiquitins to Ig-beta is required for endosomal sorting and the presentation of antigen to T cells, yet, ubiquitinylation is dispensable for receptor internalization. Membrane-bound immunoglobulin mu was not detectably ubiquitinylated nor were the conserved lysines in the mu cytosolic tail required for trafficking to late endosomes. A preliminary analysis of gene-targeted mice expressing a mutant of Ig-beta that cannot be ubiquitinylated (Ig-beta^{KdeltaR}) indicates that Ig-beta ubiquitinylation is required for normal BCR endocytic trafficking *in vivo*. These results demonstrate that ubiquitinylation of a singular substrate, Ig-beta, is required for a specific receptor trafficking event. However, they also reveal that E3 ligases play a broader role in multiple processes that determine the fate of antigen-engaged BCR complexes.

HIV-1 Vpr mediates G2 cell cycle arrest by recruiting the DDB1-CUL4A^{VPRBP} E3 ubiquitin ligase

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Human immunodeficiency virus (HIV-1) viral protein R (Vpr) is an accessory protein that mediates G2 cell cycle arrest and apoptosis in human cells. Accumulating evidence indicates that Vpr-mediated cell cycle arrest results from activation of ATR (ataxia and telangiectasia mutated and Rad3 related)-mediated checkpoint signaling through the induction of DNA replication stress. However, the mechanism by which Vpr accomplishes this function remains elusive and host cell factors directly engaged in this process remain unknown. We used tandem affinity purification to isolate native Vpr complexes. We found that damaged DNA binding protein 1 (DDB1), viral protein R binding protein (VPRBP) also known as DDB1–CUL4A-associated factor 1 (DCAF1), and cullin 4A (CUL4A) – all components of a CUL4A E3 ubiquitin ligase complex, DDB1-CUL4A^{VPRBP} – were able to associate with HIV-1 Vpr. Analysis of the complex architecture reveal that DDB1, VPRBP and Vpr form a ternary complex with VPRBP acting as a bridge between DDB1 and Vpr. Depletion of VPRBP by small interfering RNA interfered with the formation of the DDB1-VPRBP-Vpr complex and impaired Vpr-mediated induction of G2 arrest. Importantly, VPRBP knockdown alone did not affect normal cell cycle progression or activation of ATR checkpoints, suggesting that the involvement of VPRBP in G2 arrest was specific to Vpr. Interestingly, mutations in the leucine/isoleucine-rich domain of Vpr were found to impair the protein ability to interact with VPRBP and DDB1 and led to a strongly attenuated G2 cell cycle arrest. In contrast, G2 arrest–defective C-terminal Vpr mutants were found to maintain their ability to associate with these proteins, suggesting that the interaction of Vpr with the DDB1-VPRBP complex is necessary but not sufficient to block cell cycle progression. Overall, our results point toward a model in which Vpr could act as a connector between the DDB1-CUL4A^{VPRBP} E3 ubiquitin ligase complex and an unknown cellular factor whose proteolysis or modulation of activity through ubiquitination would activate ATR-mediated checkpoint signaling and induce G2 arrest.

JAMM/MPN proteins are highly specific, K63-directed deubiquitinating enzymes

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An unusual deubiquitinating activity exists in HeLa cell extracts that is highly specific for deconjugating K63-linked rather than K48-linked polyubiquitin chains. The activity is insensitive to both N-ethyl-maleimide and ubiquitin aldehyde, indicating that it does not possess an active site cysteine residue, and gel filtration experiments show that it resides in a high molecular weight (~600kD) complex. Using a biochemical approach, we found that that the K63-specific deubiquitinating activity actually corresponds to three multisubunit complexes which, remarkably, co-fractionated through six chromatographic steps. Each individual complex has K63-specific deubiquitinating activity and contains a subunit possessing a JAMM/MPN domain. The identified complexes include the 19S proteasome, the COP9 signalosome and a novel complex that includes a poorly characterized JAMM/MPN domain-containing protein called C6.1A.

We characterized the novel, C6.1A-containing complex further and determined that the K_m for K63 chain deubiquitination is ~3mM and that the activity is sensitive to both zinc chelating agents and deprotonated Tris. The C6.1A complex exclusively deconjugates K63 linkages within the context of a mixed K48- and K63-linked polyubiquitin chain, and cannot cleave K29, K6 or K11-linked chains. Both K48-linked chains and mono-ubiquitin can, however, serve as competitive inhibitors of C6.1A-dependent, K63-directed deubiquitinating activity, indicating that its specificity lies at the level of catalysis, not binding. Our results, in conjuction with those published for the protein AMSH (1), indicate that K63-specific deubiquitinating activity may be a general property of JAMM/MPN enzymes.

Recently, C6.1a was identified in a complex with BRCA1 and may function in the cellular response to DNA damage (2-4). The cytosolic complex we identified, however, is distinct from the BRCA1-associated complex, suggesting that that C6.1a may assemble into modular complexes that utilize its remarkable specificity in multiple pathways.

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Ubiquitin-mediated turn-over of HIV-1 integrase and viral gene expression

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Integration of the human immunodeficiency virus-1 (HIV-1) genome into host chromosomes constitutes a crucial step for productive infection by retroviruses. It has been recently shown that HIV-1 integrase, the viral enzyme responsible for provirus integration into the host genome, can be actively degraded by the ubiquitin-proteasome pathway. We recently identify VBP1 (von Hippel-Lindau Binding Protein 1), a subunit of the prefoldin chaperone, as a novel integrase cellular binding protein that bridges interaction between integrase and the cullin2-based VHL (von Hippel-Lindau) ubiquitin ligase. We demonstrate that VBP1 and Cullin2/VHL are required for proper HIV-1 gene expression at a step between integrase-dependent proviral integration into the host genome and transcription of viral genes. VBP1 and Cul2/VHL do not directly interfere with the transcription machinery but are rather required for HIV1 gene expression when the viral genome had been integrated through an integrase-dependent pathway. Using both an siRNA approach and Cullin2/VHL mutant cells, we show that VBP1 and the Cullin2/VHL ligase cooperate in the efficient poly-ubiquitylation of integrase and its subsequent proteasome-mediated degradation. We now propose a role for integrase degradation by the prefoldin/VHL/proteasome pathway in the integration-transcription transition of the viral replication cycle.

SV40 large T antigen binding to CUL7-FBXW8 SCF-like complex is required for cellular transformation

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SV40 large T antigen (Ag) is capable of transforming a variety of human and rodent cell types. The transforming ability of T Ag is dependent, at least in part, on binding to and inactivating several cellular tumor suppressor proteins. T Ag binds to p53 as well as the 3 members of the retinoblastoma (RB) family (pRB, p107 and p130) and serves to inactivate their growth suppressor function. In addition to RB and p53 proteins, T Ag binds to many other cellular proteins. We determined that SV40 large T Ag binds specifically to CUL7. Furthermore, we determined that T Ag could bind specifically to an SCF-like complex containing CUL7, RBX1, SKP1, and the F-box containing substrate-specificity factor FBXW8 (Fbx29 and Fbw6). To determine the role of CUL7 binding to T Ag, we generated several small in-frame deletion mutations within large T Ag and determined that the F98A, Δ98-99 and Δ69-83 mutant T Ags all failed to bind to CUL7 but retained the ability to bind to RB and p53. Importantly, these CUL7-binding defective mutant T Ags failed to transform wild type MEFs in standard transformation assays. However, each of these mutant T Ags could transform *Cul7*-null mouse embryo fibroblasts (MEFs) as well as wild type T Ag. These results indicate that CUL7 is functionally inactivated in the presence of wild-type T Ag during transformation and raises the possibility that CUL7 serves as a tumor suppressor. Similarly, we are assessing the ability of T Ag to transform *Fbxw8*-null MEFs.

CUL7 is a novel member of the cullin family of E3 ubiquitin ligases and appears to be present only in mammals. Mutations in *CUL7* gene have been reported in the 3-M human short stature syndrome. However, it is not known if CUL7 in association with FBXW8, SKP1 and RBX1 targets specific substrates for ubiquitination and subsequent degradation by the proteasome. To characterize the function of CUL7, we have identified additional binding partners. We have determined that CUL7 forms several distinct complexes; one complex resembles the canonical SCF and contains SKP1, CUL7, RBX1 and FBXW8. In addition, CUL7 forms a distinct complex with PARC and p53 that does not contain SKP1 or FBXW8. PARC is closely related to CUL7 with both proteins containing a cullin homology domain, an N-terminal CPH domain, and a central DOC domain. In addition, PARC contains a C-terminal RING-IBR (in-between-RING) domain not present in CUL7. In addition, CUL7 forms a third complex with RBX1 and GLMN that does not include p53, PARC, SKP1 or FBXW8. Thus CUL7 function appears to be highly regulated, at least in part, by the ability to form several mutually exclusive complexes. Notably T Ag can selectively bind to the SCF-like form of CUL7 containing RBX1, SKP1, and FBXW8 and not the other CUL7-containing complexes.

The ubiquitin-proteasome system facilitates HSV entry

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Herpes simplex virus (HSV) entry into cells is a multi-step process that engages the host cell machinery. Entry into a subset of host cells, including human epithelial cell lines, requires at least two cellular cues: binding to a host gD-receptor and intracellular low pH. Low pH is required for entry of Ebola and SARS coronaviruses, but acid does not directly activate membrane fusion. In these instances, viral glycoproteins are cleaved by acid-dependent, cellular cysteine proteases (cathepsins) prior to entry. We determined whether HSV entry required low pH-dependent endosomal proteases. E64 and leupeptin, which are inhibitors of cysteine proteases, had no effect on HSV entry. Together with previous studies that indicate that acid directly inactivates entry, these experiments support the hypothesis that endosomal low pH can directly activate HSV for fusion and entry. In the process of screening a panel of protease inhibitors, we determined that MG132 and lactacystin blocked HSV entry. These inhibitors of the 20S proteasome impeded entry in a concentration-dependent manner as measured by an HSV-induced reporter assay. Importantly, inhibition of the 20S proteasome blocked capsid delivery to the nucleus in the absence of gene expression as measured by confocal microscopy. This indicates that an early step in entry is proteasome-dependent. Effects on the proteasome were verified by an in vitro assay of 20S catalytic activity. The inhibitory effects of MG132 and lactacystin on the proteasome are known to be reversible and irreversible, respectively. Using wash out experiments, their inhibition of HSV entry was similarly reversible and irreversible. The 20S catalytic core of the proteasome has separable trypsin-like, chymotrypsin-like, and caspase-like proteolytic activities. Studies with inhibitors of each of the catalytic active sites indicated that the chymotrypsin-like component is specifically required for HSV entry. This site may be needed for substrate degradation during viral entry. HSV can use endocytic or non-endocytic routes to enter host cells. These distinct pathways were dependent on cellular proteasome activity, suggesting that proteasomal degradation is of universal importance, regardless of entry route. Thus, to accomplish cell entry, HSV has evolved to commandeer the two distinct cellular machineries of intracellular protein degradation: the low pH endosomal-lysosomal pathway and the ubiquitin-proteasome system.

HIV1 and 2 Vpr block cells in the G2 phase of the cell cycle by engaging a DCAF1- and cullin4A-containing ubiquitin-ligase complex

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Expression of the 14-kDa HIV1 protein Vpr in dividing cells blocks cell cycle progression in the G2 phase. Vpr also promotes low titer infections in cultures of non-dividing, terminally differentiated macrophages, modestly enhances transcription, triggers apoptosis and targets cellular uracil N-glycosylase for proteasomal degradation. HIV2 and some SIVs have two Vpr-like proteins, Vpr and Vpx. The former causes G2 arrest and the later facilitates macrophage infection. The mechanism(s) by which HIV1 Vpr achieves these functions and their relative contributions to viral replication and pathogenesis remain unclear.

We have begun to identify cellular protein partners of Vpr to establish how it modifies cellular processes. This will allow us to determine which Vpr functions are primary or secondary consequences of the interactions and to identify the mechanism(s) of Vpr action. Past work demonstrated that Vpr can contact numerous cellular proteins. In order to enhance the specificity of our search and to help determine the functions of the interactions, we focused our studies on proteins that engage HIV1 Vpr as well as either HIV2 Vpr or Vpx.

Four proteins, VprBP/DCAF1 (DCAF1), DDB1, matrin 3 and rpS3, co-immunoprecipitated with HIV1 Vpr. HIV2 Vpr engaged DCAF1 and DDB1, but not matrin 3 or rpS3. None of these proteins paired with HIV2 Vpx. We further found that Vpr assembles with DCAF1 and DDB1 into a multi-molecular complex together with cullin4A (cul4A), and uses DCAF1 as an adaptor to engage DDB1.

HIV1 and 2 Vpr both cause cell cycle arrest. We therefore tested the functional relevance of the interaction between Vpr and the VprBP/DDB1/ cul4A-containing complex by determining whether its integrity is required for Vpr-mediated G2 arrest. Vpr-mediated arrest was blocked by depletion of DCAF1, by overexpression or depletion of DDB1, or by expression of dominant negative cul4A. Unexpectedly, we also found that dominant negative cul1 blocked accumulation of cells in G2 in the presence of Vpr.

Cul1- and cul4A-containing complexes can influence cell cycle progression. We therefore examined whether depletion of DCAF1, depletion or overexpression of DDB1, or expression of dominant negative cullin1 or cullin4A interfere with the accumulation of cells in G2 either by causing marked G1 arrest or by blocking the G2 checkpoint. Only dominant negative cul1 blocked accumulation of cells in G2 in response to DNA damage.

Our data supports a model in which Vpr engages a DDB1/cul4A-containing ubiquitin ligase complex using DCAF1 as an adaptor. We hypothesize that Vpr itself acts as an adaptor, or modifies the conformation DCAF1, to recruit an ubiquitylation target to the complex. Modification of the target prevents its function in cell cycle progression.

MLV Gag acts as a functional mimic of the HECT-ligase binding protein SPG20

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Retroviruses harbor specific sequences, termed late budding domains (L-domains), in the main structural protein Gag that are essential for mediating the final stage of virus release. The murine leukemia retrovirus (MLV) is an example of a virus that possess the PPXY-type late domain, and a number of studies from our lab and others have shown that the PPXY-signal can bind to and recruit HECT ubiquitin ligases to sites of viral budding at the plasma membrane. However, how this interaction ultimately results in the recruitment of the class E vacuolar protein-sorting (VPS) pathway is unclear. We have observed that induction of class E compartments upon expression of a dominant negative form of the VPS4 protein, as well as over-expression of a number of other class E factors, causes a redistribution of certain HECT ligases to these structures. The nature of the link between these ligases and the class E machinery has yet to be established. By using yeast two-hybrid and proteomic based screens with the full length WWP1 ligase and HECT domain fragment, we have identified a number of proteins that may play a specific role in PPXY-mediated viral budding and/or interact with members of the class E VPS network. One factor, SPG20 or Spartin, bound strongly to WWP1 and other HECT ligases, via the WW-domains in a PPXY-dependent manner. Upon overexpression of SPG20, we observed dose-dependent inhibition of MLV budding, while no phenotype was seen using viruses engineered to contain a PTAP-late domain. This effect on budding was specific to SPG20, as overexpression of another PPXY-containing protein, also discovered in the yeast two-hybrid screen and binding similarly to WWP1, did not interfere with MLV budding. Finally, we were able to show that fusion of SPG20 to MLV Gag lacking a late-domain could rescue budding to levels approaching wild-type virus. From these data we believe that MLV Gag may be acting as a functional mimic of SPG20, utilizing the HECT ligases to recruit the Class E VPS factors during the budding process. The role of SPG20 is currently unknown, but mutations that prematurely truncate the protein present with a form of hereditary spastic paraplegia known as Troyer's syndrome. Interestingly, SPG20 possesses a MIT (Microtubule Interaction and Transport) domain, also found in the Class E protein Vps4. Localization of a fluorescently tagged version of SPG20 was seen as highly vesicular, and when co-expressed with WWP1 the proteins co-localized in a near-perfect manner. We performed ubiquitin-transfer experiments and found that WWP1 could specifically ubiquitinate SPG20, and that this was primarily mono-ubiquitination, similar to what we observed for MLV Gag in this assay. Surprisingly, we found SPG20 localized to the relatively uncharacterized lipid droplet organelle, and we identified protein-protein interactions with Tip47, recently proposed as a potential bridging factor between HIV Gag and Env. These data provide a framework for further investigation into the mechanisms underlying HECT ubiquitin ligase function in the context of viral budding and in the broader context of endocytosis and cargo sorting at the multi-vesicular body (MVB).

Characterization of a novel cullin5 binding domain in HIV-1 Vif

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HIV-1 Vif counteracts host restriction cytidine deaminase (APOBEC3G) A3G by co-opting the cellular ubiquitin-proteasome machinery. Vif utilizes a viral specific BC-box to recruit ElonginB-ElonginC and a novel zinc binding HCCH motif to recruit Cullin5 (Cul5) to form an E3 ubiquitin ligase targeting A3G for polyubiquitination and subsequently proteasomal degradation. To further determine the structural requirements in HIV-1 Vif HCCH motif for Cul5 binding and Vif function, we investigated the arrangement of the His and Cys residues, the role of the spacing between them, and the requirement for the conserved residues. Our data demonstrated that exchanging Cys for His and vice versa in the highly conserved Zn-coordinating HCCH motif disrupted Vif function and interaction with Cul5. Moreover, the maintenance of both conserved residues and spacing within the HCCH motif is critical for Vif function. We have identified a 'viral Cul5 box' with consensus Hx2YFxCFx4Φx2AΦx7-8Cx5H that is required for Cul5 selection and subsequent A3G degradation. This novel motif may represent a potential new target for anti-viral drug development.

CUL7 recruitment to SV40 T antigen promotes proteolytic destruction of DNA damage signaling proteins

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Although the DnaJ chaperone activity of SV40 T antigen (Tag) is not required for viral DNA replication in cell extracts, mutations that inactivate it cripple viral DNA replication in infected cells. The J domain (aa5-102) consists of 4 conserved alpha helices linked by long loops. It binds to at least 3 host proteins: Hsc70, the SCF ubiquitin ligase subunit CUL7, and the mitotic checkpoint protein Bub1. How these functions promote SV40 replication in cells remains poorly understood. Human and monkey cells infected with wild type SV40 develop an ATM-mediated DNA damage response, as detected by confocal immunofluorescence microscopy and Western blot analysis of total cell extracts at different times after infection. Host proteins that undergo phosphorylation during infection include ATM itself, Nbs1, p53, and Chk2, but not Chk1. A variety of host DNA damage response proteins, e.g. Mre11-Rad50-Nbs1 (MRN), are re-localized into subnuclear foci as the infection progresses. Steady-state levels of MRN subunits decline to differing extents depending on the host cell. Quantitative RT-PCR indicates that MRN mRNA levels remain unchanged, but the proteasome inhibitor MG132 stabilizes the MRN complex, implying that MRN is targeted for destruction. CUL7 redistributes in cells expressing wild type Tag from the cytoplasm to a nuclear/perinuclear location. Large nuclear foci containing Tag, MRN, gamma-H2AX, DNA pol alpha, and conjugated ubiquitin assemble in infected cells, suggesting that viral DNA replication and MRN degradation may take place in these compartments. Tag mutations (F98A, del69-82) that weaken CUL7 binding eliminate oncogenic transformation of mouse cells by SV40. However, F98A virus is viable while the deletion mutant is not. We found that primate cells infected or transfected with the F98A or del69-82 mutants induce a host DNA damage response similar to that of the wild type virus, but CUL7 does not re-localize and MRN does not undergo degradation. Taken together, our results indicate that SV40 Tag induces degradation of host DNA damage-signaling proteins, that this activity depends on its ability to bind CUL7, and that degradation of MRN may be one of the J domain functions that facilitates viral DNA replication. The properties of other point mutations in the J domain are under investigation. (Supported by NIH GM52948, HHMI Professors Program, and Vanderbilt University)

Degradation by the Ad5 E4orf6 E3 ligase complex is required for AAV5 replication

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Full replication of adeno-associated virus type 5 (AAV5) is sustained by adenovirus type 5 (Ad5) helper functions EIA, EIB, E2a, E4Orf6 and VA RNA; however, their combined net enhancement of AAV5 replication was comprised of both positive and negative individual effects. Although Ad5 E4Orf6 was required for AAV5 genomic DNA replication, it also functioned together with EIB, to degrade *de novo* expressed, pre-assembled AAV5 capsid proteins and Rep 52 in a proteosome-dependent manner. VA RNA enhanced accumulation of AAV5 protein, overcoming the degradative effects of E4Orf6, and was thus required to restore adequate amounts of AAV5 proteins necessary to achieve efficient virus production.
The degradative effects of E4Orf6 showed limited target specificity and required the presence of both BC Box motifs to bring about degradation of capsid proteins. E4Orf6 formed an E3 ligase complex, and complexing of E4Orf6 to AAV5 capsid proteins took place only in the presence of E1b-55k. E4Orf6-directed degradation of AAV5 capsid proteins required Cullin 5 and E4Orf6 mediated ubiquitin chain elongation onto pre-assembled capsid proteins. The degradative effect of E4Orf6 was required for AAV5 replication, and while VA RNA was required to restore AAV5 protein to levels necessary for efficient infection, E4Orf6 had a role in viral replication that could not be complemented by VA RNA.

Role of macrophage phagocytosis of amyloid-beta in Alzheimer Disease

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Amyloidosis of the brain is the principal factor in Alzheimer disease (AD) pathogenesis. Patients with AD have defective innate immunity with respect to amyloid-beta (Abeta) clearance. Our molecular studies show that deficient transcription of genes related to glycosylation, cell adhesion, and innate immunity including antiviral defense and Toll-like receptors. The critical gene N-acetylglucosaminyltransferase (GnTIII) (which is coded by MGATIII) is severely downregulated in a majority of patients with AD in the age group 60 – 80 years. This gene plays critical role in macrophage function since blockade of its transcription by MGATIII siRNA inhibits phagocytosis of Abeta by macrophages and other functions of macrophages. Trafficking of amyloid-beta in macrophages of AD patients is severely limited to surface binding but usually no passage to late endosomes and lysosomes. Thus the problems with disposal of viruses and misfolded proteins may find some common and distinct mechanisms.

Epstein-Barr nuclear antigen 1 destabilizes p53 and PML nuclear bodies in nasopharyngeal carcinoma cells through interactions with USP7

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Epstein-Barr virus is a common herpesvirus that immortalizes host cells as part of its latent mode of infection and, as a result, is associated with several types of cancer, in particular Burkitt's lymphoma and nasopharyngeal carcinoma (NPC). EBNA1 is the only viral protein required to maintain latent infection in proliferating cells and the only viral protein expressed in all types of EBV-induced cancers. We have previously used proteomics approaches to profile the cellular protein interactions of EBNA1 and discovered a stable interaction with the host ubiquitin specific protease, USP7, which modulates the p53 pathway by stabilizing p53 and Mdm2. EBNA1 inhibits USP7 binding to p53 and Mdm2 *in vitro* by competing for the same binding pocket in the USP7 N-terminal domain, leading to an anti-apoptotic effect under experimental conditions (Saridakis et al 2005 Mol Cell 18, 25; Sheng et al 2006 Nature Struc. Mol. Biol. 13, 285). Recently, we have begun to examine the cellular effects of EBNA1 in a naturally occurring EBV-tumour, NPC. NPC cells tend to lose the EBV when grown in culture but can be engineered to stably express EBNA1. EBNA1 expression in these cells results in reduced levels of both p53 and Mdm2 as well as a notable reduction in the number of PML nuclear bodies and the amount of PML protein. Silencing of EBNA1 with siRNA in NPC cells expressing EBNA1 results in restoration of the levels of PML protein and nuclear bodies. Transient expression of EBNA1 in NPC cells also resulted in the destruction of PML nuclear bodies and PML protein in a dose-dependent fashion and was used to assess various EBNA1 mutants for sequences important for this effect. Unlike wildtype EBNA1, the EBNA1 mutant lacking the USP7-binding sequence had no obvious effect on PML nuclear bodies suggesting that EBNA1 binding to USP7 is important for PML destabilization. In keeping with previous reports, we found USP7 to be partly associated with PML nuclear bodies in NPC cells and, in a subset of EBNA1-expressing cells, EBNA1 itself was seen to localize to PML nuclear bodies. The results show that EBNA1 disrupts PML nuclear bodies in some epithelial cells and implicate the EBNA1-USP7 interaction in this process.

Identification of a role for the GGA and Arf proteins in retroviral assembly and release

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Retroviral Gag proteins are the major structural determinant of virus particle assembly and release. Although the domains in Gag that direct the late stages of retroviral replication are now well defined, little is known about the cellular factors that regulate the trafficking of Gag to the subcellular site(s) of virus assembly. In this study, we identified the Golgi-localized gamma ear-containing Arf-binding (GGA) proteins and the ADP ribosylation factors (Arfs) as cellular modulators of retrovirus particle production. The GGAs (GGA1, GGA2, and GGA3) are a family of clathrin adaptors that regulate protein trafficking between Golgi and endosomal membranes. We observed that GGA overexpression led to a marked inhibition in the assembly and release of human immunodeficiency virus type 1 (HIV-1), equine infectious anemia virus (EIAV), and murine leukemia virus (MLV) particles. Biochemical analyses revealed that GGA overexpression impaired the membrane association of Gag and domain mapping experiments demonstrated that overexpression of the GAT (GGA and TOM) domain of the GGAs was sufficient to potently inhibit virus particle production. The GAT domain encompasses binding sites for the Arf proteins, Tsg101 and ubiquitin and directs GGA binding to membrane. Immunofluorescence microscopy indicated that GGA overexpression led to the accumulation of aberrant swollen endosome-like compartments in cells, which sequestered ubiquitinated cargo. It is well established that a number of retroviral Gag proteins are ubiquitinated and, interestingly, we observed that GGA1-induced compartments also trapped newly assembled virus particles. Moreover, exogenous overexpression of ubiquitin abolished the accumulation of the GGA-induced compartment and rescued the virus production defect imposed by GGA overexpression. Consistent with a connection between GGA overexpression and Arf activity, we observed that GGA overexpression led to Arf sequestration, and inhibition of particle production imposed by GGA overexpression was lost upon mutation of the Arf-binding sites in GGA2 and GGA3. Finally, disruption of endogenous Arf activity inhibited particle production by decreasing Gag–membrane binding, and siRNA-mediated depletion of class I and class II Arfs inhibited HIV-1 assembly and release. These findings identify the GGA and Arf proteins as novel modulators of retroviral Gag trafficking to the plasma membrane.

Looking for new potential DUBs within EBV ORFeome

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Ubiquitin and ubiquitin-like proteins are signalling messengers that regulate many cellular functions in addition to protein degradation, such as cell proliferation, apoptosis, transcription regulation, the cell cycle and DNA repair. Manipulation of the ubiquitin proteasome system (UPS) is emerging as a common theme in viral pathogenesis. There are now enough examples on functional homologs of UPS enzymes encoded in some of the viral genomes suggesting that a systematic identification of these viral products may provide new insights into virus-host-cell interactions.
Deubiquitinating enzymes (DUBs) regulate the activity of the UPS by hydrolyzing ubiquitin isopeptide bonds and are emerging as critical enzymes in the cascade. DUBs are classified into five different subfamilies: USPs, UCHs, Otubains, Josephines and JAMMs, JAMMs are metalloproteases while others belong to cysteine protease family and share conserved catalytic Cys and His domains. We developed both a bioinformatics strategy and biochemical chemical assay to screen the genome of human gamma herpes virus, Epstein-Barr Virus (EBV). We cloned all the EBV open reading frames (ORFeome) in to bacterial expression vector to test them *in vivo.* First we have applied bioinformatics strategy to predict putative DUBs in the genome. Sequence based search against 106 ORFs covering the entire genome of EBV resulted in a set of candidates that contain putative C and H-boxes. One of the candidates identified is BPLF1 ORF, which was earlier shown to have isopeptidase activity. Further we are validating these candidates for DUB activity by *in vivo* assay.

Viral OTU domains: A new class of immune evasion proteases targeting both ubiquitin and ISG15 conjugates

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Ubiquitin and the ubiquitin-like protein ISG15 are critically important in innate immune responses mediated by tumor necrosis factor-alpha (TNF-alpha) and type I interferon (IFN alpha/beta). Ubiquitination regulates NF-kB activation and ISG15 is a potent IFN-induced antiviral molecule. As viruses have evolved immune evasion mechanisms to counteract anti-viral molecules, it is likely that ubiquitin and ISG15 will be targets of immune evasion strategies. The ovarian tumor (OTU) domain is a cysteine protease domain found in eukaryotic, prokaryotic and viral proteins. Although the majority of the OTU-domain containing proteins remain uncharacterized, several OTU-domain mammalian proteins have deubiquitinating (DUB) activity. As we had evidence that an OTU-domain containing protein could bind ISG15, we hypothesized that viral OTU domains were novel immune evasion proteases with deubiquitinating and deISGylating activity

Viral OTU domains were cloned from the large (L) protein of nairoviruses CCHFV and DUGV, and from the nsp2 protein of arteriviruses EAV and PPRSV. Expression of viral OTU domains resulted in an overall decrease of cellular ubiquitin and ISG15 conjugates. Using deletion mutants from CCHFV-L, the deconjugating activity was mapped to the OTU domain and confirmed by mutating the predicted protease active site residues which restored ubiquitin and ISG15 conjugate levels. *In vitro* deconjugation assays demonstrated that the CCHFV-L OTU domain could cleave K48- or K63-linked ubiquitin chains and ISG15 conjugates. SUMO-2 or SUMO-3 chains were not deconjugated, suggesting that the CCHFV-L OTU domain may have specificity for certain UB/UBL family members.

The physiologic importance of viral OTU domains was assessed in reporter assays, an OTU domain-expressing transgenic mouse and by generating chimeric Sindbis viruses. Transfection of CCHFV-L or EAV-nsp2 OTU domains decreased TNF-alpha induced NF-kB activation. A transgenic mouse expressing the CCHFV-L OTU domain displayed increased susceptibility to Sindbis virus infection. Since expression of ISG15 from a recombinant Sindbis virus increases survival of infected *Ifnar*$^{-/-}$ mice, we hypothesized that OTU-mediated deISGylation should abolish ISG15's anti-viral function. Co-expression of the OTU domain antagonized ISG15's anti-viral function, while expression of a catalytic mutant OTU domain had no effect on ISG15-mediated protection from lethality. The ability of viral proteins to deconjugate both ubiquitin and ISG15 modified proteins could potentially represent a powerful immunomodulatory mechanism which may allow viruses to affect the regulation of numerous cellular pathways.

Nuclear pore complex-associated SUMO isopeptidase functions as a karyopherin-alpha releasing factor

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SUMO (Small Ubiquitin-related Modifiers) are dynamic and reversible post-translational modifiers that regulate numerous cellular functions. Multiple lines of evidence indicate that SUMO modification is involved in nucleocytoplasmic transport. In particular, SUMO conjugating and deconjugating enzymes localize to nuclear pore complexes (NPCs). Intriguingly, the localization of a SUMO specific isopeptidase at NPCs is conserved among organisms ranging from yeast to mammals. However, the functions of SUMO isopeptidases, and SUMO modification in general, at NPCs are poorly understood. We have examined the functional significance of the localization of the mammalian isopeptidase SENP2 to NPCs in order to gain insight into the function of SUMO modification in nucleocytoplasmic transport. The yeast homolog of SENP2, Ulp1, is tethered to NPCs through interactions with soluble transport receptors, karyopherin-alpha and beta and Pse1; however, little is known about the mechanism by which SENP2 is targeted to NPCs. We have identified two targeting domains within the N-terminus of SENP2 that cooperatively mediate its localization to the nucleoplasmic face of NPCs. One of these domains contains a karyopherin-alpha binding site (NLS) that mediates direct interaction with karyopherin-alpha *in vitro* and *in vivo*. Unlike in yeast, however, this interaction is not required for SENP2 localization to NPCs. Instead, we show that SENP2 interacts with karyopherin-α through this high affinity NLS, and displaces the SV-40 NLS, a classical NLS-cargo. This finding indicates a role for SENP2 in coordinating the disassembly of karyopherin-alpha and beta transport complexes at the nucleoplasmic face of the NPC, implicating it as a member of the family of karyopherin-alpha releasing factors (KaRFs). Studies have been initiated to identify SUMO-modified proteins recognized by SENP2 at NPCs and to determine whether members of the karyopherin-alpha and beta complex or cargo proteins are targets for SUMO deconjugation at NPCs. By elucidating the functional significance of SENP2 localization to the NPC, these studies will provide insight into the role of SUMO modification in regulating nucleocytoplasmic transport.

Microtiter plate assays for the assessment of biochemical activities of E2 and E3 ubiquitin enzymes

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The ubiquitin-proteasome pathway plays a major role in cancer development and is a bona fide target for cancer therapy. The covalent attachment of ubiquitin to a target protein proceeds through a multi-enzyme cascade. Initially, E1 activates ubiquitin and is then transferred to a cysteine residue of an E2 ubiquitin-conjugating enzyme (ubc). Finally, the E2 itself, or more commonly in concert with an E3 ubiquitin ligase, ligates the ubiquitin via its carboxy terminus to lysine residues of a protein substrate. This enzymatic cascade, responsible for ubiquitylating target proteins is complex, and its regulation is only beginning to be understood. The complexity stems from the large number of E2 and E3 enzymes; in humans, more than 30 E2s and hundreds of putative E3 ligases have been identified. The enzymatic nature, multitude of E2 and E3s and their specific substrate recognition predestines them as therapeutic targets in major diseases.

Targeting components of ubiquitination pathway for drug discovery requires high-throughput assays. Here, we developed 96-well assays for measurement of activities or inhibitor screening of various E2 and E3 enzymes. We present assays for measuring activities of the E2 enzymes UbcH2, UbcH3 (cdc34), UbcH5c, Ubc13/Uev1 and of the E3 enzyme MDM-2 employing biotinylated ubiquitin and subsequent detection with streptavidin-HRP. Furthermore, in vitro ubiquitination experiments of important client proteins such as p53 or histones were performed. Ubiquitinated protein substrates or ubiquitin enzymes were specifically captured through immobilized antibodies or Nickel Chelate coated microwell plates.

We show that the small molecule ubiquitination inhibitor Ro106-9920 inhibited ubiquitination of various substrate proteins by blocking E2 and/or E3 activity in a dose-dependent manner. We demonstrate that some E2 enzymes directly catalyze isopeptide bond formation between ubiquitin and substrate proteins in vitro. The E2 enzyme UbcH5, but not UbcH7, efficiently ubiquitinated recombinant p53, c-myc, and p27. Conversely, UbcH5 did not ubiqutinate histone 1.2 or heat shock protein 90a. However, UbcH2 efficiently ubiquitinated histone 1.2 as evidenced by immunoblot and 96-well assays. A hallmark of E3 ligases is their capability to ubiquitinate itself, therefore we also designed an assay for E3 ligase self-ubiquitination. Using recombinant and active MDMD2, a RING-finger-type E3 ligase, we show that in concert with E1 and E2 enzymes MDM2 is self-ubiquitinated and this event was inhibited by low micromolar concentrations of Ro106-9920.

In conclusion, the speed and convenience of the above assay formats provides a useful platform for screening of various compounds affecting E2 and E3 enzymes. Furthermore, the assay format permits assessment of ubiquitination of protein substrates in an ELISA-type fashion.

ICP0 antagonizes STAT 1-dependent repression of herpes simplex virus: Implications for the regulation of viral latency

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ICP0 is an E3 ubiquitin ligase that can trigger the reactivation of latent herpes simplex virus type 1 (HSV-1). ICP0 is encoded in the HSV-1 latency-associated locus, and appears to serve as an 'ON switch' in the HSV-1 life cycle. It is not evident how ICP0 fulfills this function in vivo. The current study was initiated to determine if ICP0's role as a viral interferon (IFN) antagonist may be relevant in this regard. Unlike wild-type (ICP0+) virus, the productive replication of ICP0-null viruses was rapidly repressed in scid or rag2-/- mice, and these animals remained healthy for months after inoculation. Rag2-/- mice that lacked the IFN-alpha/beta receptor or a downstream signaling protein, Stat 1, failed to repress ICP0-null viruses and succumbed to infection. Thus, failure to synthesize ICP0 allows the innate immune response to stably repress HSV-1 replication in vivo. These results may be relevant to viral latency because the only proteins encoded from the HSV-1 latency-associated locus, ICP0 and ICP34.5, are both IFN antagonists. ICP0-null viruses, which were avirulent in scid mice, induced an immune response in wild-type mice that was highly protective against lethal challenge with HSV-1. Thus, IFN-sensitive ICP0-null viruses appear to possess the safety and efficacy profile of a live vaccine against herpetic disease.

The role of WWP1-Gag interaction and Gag ubiquitination in assembly and release of HTLV-1

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The PPPY motif in the matrix (MA) domain of human T-cell leukemia virus type 1 (HTLV-1) Gag associates with WWP1, a member of the HECT domain containing family of E3 ubiquitin ligases. Mutation of the PPPY motif arrests particle assembly at an early stage and abolishes ubiquitination of MA. Similar effects are seen when Gag is expressed in the presence of a truncated form of WWP1, which lacks the catalytically active HECT domain (C2WW). To understand the role of ubiquitination in budding, we mutated the four lysines in MA to arginines, and identified lysine 74 as the unique site of ubiquitination. Virus-like particles produced by the K74R mutant did not contain ubiquitinated MA, and showed a fourfold reduction in the release of infectious particles. Furthermore, the K74R mutation rendered assembly hypersensitive to C2WW inhibition; K74R Gag budding was inhibited at significantly lower levels of expression of C2WW compared with wild type Gag. This finding indicates that the interaction between Gag and WWP1 has additional functions in particle assembly and release beside Gag ubiquitination. Additionally, we show that the $PPPY^-$ mutant Gag exerts a strong dominant negative effect on the budding of wild type Gag, further supporting the importance of recruitment of WWP1 to achieve particle assembly.

Viral immune evasion protein mK3 targets MHC I proteins for ER associated degradation by ubiquitination of the cytoplasmic tail of the heavy chain via serine, threonine or lysine residues

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The immune evasion protein mK3 is a viral RING-CH type E3 ubiquitin ligase encoded by murine gamma herpesvirus68. mK3 specifically targets nascent MHC I heavy chain (HC) for rapid degradation in a ubiquitination-proteasome dependent manner, thus blocking detection of virally infected cells by CD8 T cells. Interestingly TAP is the primary binding partner of mK3 and this association is required for mK3-mediated degradation of HC. To address the question of how HC is retrotranslocated from the ER and what role mK3 plays, we investigated ubiquitin conjugation sites in HC using mutagenesis and biochemistry approaches. Surprisingly, we found that a totally K-less HC had a similar polyubiquitination pattern and rapid degradation as wt HC in mK3 expressing cells. Furthermore, we found that polyubiquitinated HCs became undetectable after removal of their cytoplasmic tails by proteinase K digestion or thrombin cleavage at an engineered site. HCs with tail substitutions to remove all K, C, S & T residues abolished mK3-mediated polyubiquitination and ERAD, whereas adding back single S, T, or K residues to the tail restored polyubiquitination and ERAD. Also, the location of S, T or K residues was found to be important in that efficacious ubiquitination occurred at the sites toward the C terminus of the HC tail and not at the site proximal to the membrane. Chemically, the polyubiquitination via S or T residues was labile to mild alkali treatment suggesting an ester bond linkage between S or T and ubiquitin. Overall, these findings indicate a novel chemical mechanism of ubiquitination. In addition, our data strongly supports a model in which the binding of mK3 to TAP brings the RING-CH domain of mK3 into the proper orientation/ proximity with the tail of HC, facilitating the ubiquitination of tail S, T, or K residues and subsequently to vectorial dislocation from the ER membrane to the cytosol for degradation.

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Cellular changes in HeLa cells undergoing senescence maturation induced by inhibition in HPV E6/E7 oncogene expression

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Inhibition of HPV18 E6 and E7 expression by means of the E2 protein of BPV1 has been shown to induce a state of cellular senescence in HeLa and other HPV-positive cervical carcinoma cells. This suggests a role of the viral oncoproteins in preventing the host cells from entering a senescent state. Previously, we reported that, at an early stage of E6/E7 depletion, the growth-arrested HeLa cells are able to be stimulated to resume proliferation suggesting a reversible growth-regulation state, but at later periods, they get trapped in a irreversible growth-arrest state. This indicates that, upon depletion of the viral oncoproteins, a senescent state is irreversibly induced in HeLa cells after a period of commitment, which we previously termed 'senescence maturation'. In this study, we focused on the cellular changes taking place during the senescence maturation. During 2-5 day period post E2 transduction, there was initial drop in the telomerase activity at day 2 (10 % of the mock-treated). And, in 5 days, the activity was totally abolished due to lack of hTERT expression. In day 2, reexpression of E6 and E7 together or individually induced hTERT expression, but in day 5, cells could not express hTERT in the presence of these proteins. These results suggest that the senescence maturation is accompanied by changes in the activities of certain key host factors regulating cell proliferation. We constructed a HeLa-derivative cell line that express hTERT form CMV IE promoter. Although, telomerase activity was well maintained in these cells, transduction of E2 still prevented them from synthesizing DNA, and eventually induced cellular senescence. These results indicate as expected that, although hTERT activation is regulated differently during senescence maturation, the telomerase activity by itself is not sufficient in preventing cells from entering senescence. We also examined the status of other cellular factors in cells under prolonged state of senescence, and the results will be discussed.

HIV-1 accessory protein Vpr function is mediated by its association with the damaged DNA binding protein, DDB1

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The HIV-1 accessory protein, Vpr, localizes to the nucleus of infected cells and is packaged into virions through its association with the carboxy-terminal P6 portion of Gag. It is not required for virus replication and only provides a modest boost to replication in primary T cells. Vpr arrests the cell cycle of infected cells in G_2. The protein induces apoptosis and provokes a cellular response that resembles that of DNA damage, with the activation of ATM/ATR and formation of γH2AX foci. Using tandem affinity purification pull-down and mass spectrometry we have identified two cellular proteins that bind to Vpr. One is the damaged DNA binding protein 1 (DDB1). The other is the previously identified Vpr binding partner, Vpr binding protein (VPRBP), which has been, recently renamed DDB1 cullin 4A associated factor 1 (DCAF1). DDB1, in association with CUL4A, ROC1 and a series of adaptor proteins forms a chromatin-associated E3 ubiquitin ligase that mediates the ubiquitination of a variety of cellular substrates and is activated during DNA replication and DNA damage. Interestingly, DDB1 is targeted by the viral proteins SV5 V and hepatitis virus X to block type I interferon responses. Vpr induced G_2 arrest and apoptosis required the interaction with DCAF1/DDB1. Vpr bound to the DNA repair enzyme, uracil DNA glycosylase, inducing its degradation by association with DDB1/CUL4A. Vpr interfered with the repair of UV light damaged DNA through its association with the DDB1/CUL4A E3 ubiquitin ligase. Our findings suggest that Vpr induces the degradation of cellular proteins perhaps as a means of ensuring a favorable cellular state for its replication and avoiding antiviral host proteins.

Biochemical characterization of the retroviral Restriction Factor TRIM5a

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In certain primate species, specific alleles of the protein TRIM5a potently inhibit retrovirus replication at early stages of the viral life cycle, indicating that TRIM5a represents an important intrinsic defense against cross-species viral transmission (Stremlau et al. Nature. 2004). TRIM5a restricts viral replication by a mechanism that involves viral capsid binding and the inhibition of subsequent reverse transcription and nuclear integration steps. Collective evidence suggests that TRIM5a interacts directly with the viral capsid, but this has yet to be definitively demonstrated experimentally due to inherent difficulties encountered in preparing both purified TRIM5a and viral capsids (Stremlau et al. PNAS. 2006).

TRIM5a is a member of the tripartite motif family of proteins and contains RING, B-BOX, coiled-coil and SPRY domains. The RING domain of several TRIM isoforms, including TRIM5a, functions in autoubiquitylation and in protein turnover in vivo (Diaz-Griffero et al., 2006). A chimeric TRIM5 isoform containing the RING domain of TRIM21 is less rapidly turned over and is more amenable to exogenous expression in multiple eukaryotic cell types (Li et al. J. Virology. 2006). Evidence suggests that the TRIM5a SPRY domain is the principal determinant of both viral capsid binding and the species-specificity of TRIM5a restriction (Stremlau et al. Nature. 2004.; Stremlau et al. PNAS. 2006.; Perez-Caballero et al. J Virology. 2005.; Ohkura et al. J Virology. 2007.)

To elucidate the process of TRIM5a restriction further, a TRIM5a chimera containing the RING domain of TRIM21 was expressed and purified using recombinant baculovirus and SF-21 insect cells. HIV-1 CA-NC and HIV-1 CA assemble *in vitro* under high salt to form conical and tubular structures containing the hexameric CA lattice found in authentic viral cores. Purified $TRIM21_{RING}5a$ bound directly to these synthetic viral particles in a centrifugation and sucrose cushion-based binding assay. Purified $TRIM21_{RING}5a$ also inhibited the assembly of these particles as assayed using 350nm light scattering and electron microscopy to monitor HIV-1 CA-NC assembly.

The work described here demonstrates that a purified TRIM5a protein binds directly to HIV viral capsid assemblies and also reveals that purified TRIM5a inhibits CA-NC assembly, consistent with the idea that TRIM5a binding can destabilize the viral capsid.

Identification of small molecule inhibitors of USP18 with a novel isopeptidase assay

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ISG15 is an ubiquitin-like protein (UBL) that can be conjugated to lysine residues of target proteins. UBLs can be removed from target proteins by a class of enzymes called isopeptidases and these enzymes demonstrate selectivity for specific UBLs. The functional impact of protein ISGylation is not well understood but previous experiments suggest ISG15 plays a role in the immune system and possibly cancer. Consistent with a role in immunity, RNAi directed at USP18, an ISG15-specific isopeptidase, leads to a decrease in viral replication in infected cells. In addition, the SARS virus produces an isopeptidase capable of removing ISG15 from target proteins again suggesting a role in viral infection/immunity. We have developed a novel, high throughput assay capable of measuring isopeptidase activity directed at UBLs. We expressed and purified recombinant USP18 and its mouse homolog, UBP43, using affinity chromatography. We have determined that both of these isopeptidases selectively target ISG15 conjugates. In contrast, the SARS viral protease, papain-like protease, exhibits both deubiquitinating and deISGylating activity. We have screened a library of small molecules representing diverse chemical structures. In order to determine selectivity and gain insights into the mechanism of compound action we have expressed ~65 human ubiquitin and ubiquitin-like isopeptidases. These isopeptidases, which include deneddylases, deubiquitinases, and desumoylases, will be used to determine the selectivity of identified small molecules. Interesting compounds are being developed as potential therapeutic agents in cell based assays mimicking viral infection.

Structure and function of flavivirus NS5 methyltransferase

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The plus-strand RNA genome of flavivirus contains a 5' terminal cap 1 structure (m^7GpppAmG). The flaviviruses encode one methyltransferase, located at the N-terminal portion of the NS5 protein, to catalyze both guanine N-7 and ribose 2'-OH methylations during viral cap formation. Representative flavivirus methyltransferases from dengue, yellow fever, and West Nile virus (WNV) sequentially generate GpppA-->m^7GpppA-->m^7GpppAm. The 2'-O methylation can be uncoupled from the N-7 methylation, since m^7GpppA-RNA can be readily methylated to m^7GpppAm-RNA. Despite exhibiting two distinct methylation activities, the crystal structure of WNV methyltransferase at 2.8 Å resolution showed a single binding site for S-adenosyl-L-methionine (SAM), the methyl donor. Therefore, substrate GpppA-RNA should be repositioned to accept the N-7 and 2'-O methyl groups from SAM during the sequential reactions. Electrostatic analysis of the WNV methyltransferase structure showed that, adjacent to the SAM-binding pocket, is a highly positively charged surface that could serve as an RNA binding site during cap methylations. Biochemical and mutagenesis analyses show that the N-7 and 2'-O cap methylations require distinct buffer conditions and different side chains within the K61-D146-K182-E218 motif, suggesting that the two reactions use different mechanisms. In the context of complete virus, defects in both methylations are lethal to WNV; however, viruses defective solely in 2'-O methylation are attenuated and can protect mice from later wild-type WNV challenge. The results demonstrate that the N-7 methylation activity is essential for the WNV life cycle and, thus, methyltransferase represents a novel target for flavivirus therapy.

The PLpro domain of SARS-CoV disrupts innate immunity by inhibiting the activation of IRF-3

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Severe acute respiratory syndrome coronavirus (SARS-CoV) is a novel coronavirus that causes a highly contagious respiratory disease, SARS, with significant mortality. While factors contributing to the highly pathogenic nature of SARS-CoV remain poorly understood, infection of cultured cells with SARS-CoV does not induce the production of type I interferons (IFNs), one of the major immediate antiviral responses to many viral infections, suggesting that SARS-CoV has evolved to evade or counteract innate host defenses. Recently, the papain-like protease (PLpro) of SARS-CoV was shown to have deubiquitination activity, in addition to its role in directing the processing of viral nonstructural proteins. As ubiquitination plays a pivotal role in many cellular processes including innate immunity signaling, we explored the effect of PLpro on the induction of cellular IFN response. Ectopic expression of the SARS-CoV PLpro domain dose-dependently inhibits the activation of IFN-beta promoter and induction of IFN-stimulated genes through either Toll-like receptor 3 or the retinoic acid inducible gene I/melanoma differentiation associated gene 5, the two major innate antiviral signaling pathways. The disruption of IFN response by PLpro does not require the protease activity of PLpro. Rather, PLpro interacts with IFN regulatory factor-3 (IRF-3) and inhibits its phosphorylation, nuclear translocation and association with the transcriptional co-activator CBP, thereby disrupting its function in activating the transcription of type I IFNs and other IRF-3 target genes. Our data suggest that regulation of IRF-3-dependent antiviral defenses by PLpro may contribute to the establishment of SARS-CoV infection.

A Viral ubiquitin ligase prevents the accumulation of DNA repair proteins at sites of cellular damage

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Infection with Herpes Simplex Virus (HSV-1) induces a cellular DNA damage response that aids viral replication. In neuronal cells, which are less able to mount this response, HSV-1 replication is stalled and a latent infection is established. In contrast, during infection of non-neuronal cells, many DNA repair proteins accumulate at sites of viral DNA replication and the lytic cycle ensues. The viral protein ICP0 is an E3 ubiquitin ligase that is required for the switch between lytic and latent infection. We have observed that expression of ICP0 can prevent the accumulation of cellular DNA repair factors at irradiation-induced foci (IRIF). The effect is dependent on the RING finger domain of ICP0 but proteasome-mediated degradation is not required. The ICP0-induced block to IRIF is upstream of 53BP1 recruitment and appears to be at the level of histone modifications. We propose that ICP0 induces the K63-linked ubiquitination of a cellular chromatin target to prevent the immobilization of DNA repair proteins at IRIF.

Combination of proteasome and HDAC6 inhibitors for therapy of uterine cervical cancer

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The oncogencity of Human papillomavirus E6 is mediated in large part by targeting p53 and PDZ-family tumor suppressor proteins for proteasomal degradation. PS-341 (bortezomib) is an inhibitor of the proteasomal pathway and is approved for the treatment of multiple myeloma and a phase II trial in cervical cancer is ongoing. The proteasomes mediate degradation of 85% of intracellular proteins whereas the lysosomal pathway is mostly responsible for degradation of extra-cellular proteins. However there is an exception to this rule. Under ubiquitin-proteasome system (UPS) stress (for example in presence of proteasome inhibitors and frequently in cancer cells), the lysosomal pathway becomes a buffer for degradation of redundant, off-pathway intracellular proteins that the proteasomal system was unable to clear. Thus this lysosomal pathway is accessory to proteasomal degradation. The cytoplasmic histone deacetylase 6 (HDAC6) is critical to degradation of misfolded, off-pathway poly-ubiquitinated proteins as a compensatory mechanism in response to impaired ubiquitin proteasome system activity. Our immunohistochemical analysis of HDAC6 shows a dramatic increase in expression in CIN and cervical carcinoma as compared to normal cervical epithelium. We hypothesized that the HDAC6 lysosomal pathway of protein degradation renders proteasome inhibitors less effective as cervical cancer therapeutics and that small molecule inhibitors of each pathway (PS-341 and Trichostatin A (TSA)) will act synergistically. In the present study, we find that cervical cancer cell lines exhibit greater sensitivity to proteasome inhibitors than HPV-negative cervical cancers or primary human keratinocytes. Treatment of cervical cancer cells with PS-341 dramatically elevated the level of p53 but not hDlg, hScribble or hMAGI. Importantly the combination of PS-341 and HDAC inhibitor TSA or the HDAC6 inhibitor tubacin show synergistic killing of HPV-positive cervical cancer cell lines in association with high levels of p53 and acetylated tubulin. Similarly, treatment of immunodeficient mice bearing HeLa tumors with the combination of PS-341 and TSA was able to control tumor growth significantly more effectively than either agent alone. In sum, combination of PS-341 and HDAC6 inhibitor warrants exploration for the treatment of cervical cancers and other HPV-related disease.

Components of VICE domains appear to play active roles in productive HSV-1 infection

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Productive HSV-1 infection results in the formation of nuclear replication compartments in which replication and encapsidation of the HSV-1 DNA genome take place. Cellular proteins involved in protein remodeling such as the 20S proteasomal subunit, chaperone proteins such as Hsc70 and ubiquitin are reorganized into Virus-Induced Chaperone-Enriched (VICE) domains that form adjacent to replication compartments in infected cells. VICE domains also contain misfolded proteins and thus share several characteristics with cellular aggresomes: inclusion bodies that sequester misfolded protein and protein remodeling machinery. In order to determine whether or not the 20S proteasomal subunit is catalytically active in VICE domains, uninfected and infected human fibroblasts were microinjected with a 20S proteasomal substrate that fluoresces green upon degradation. Flourescent foci of proteasomal activity were observed in the nuclei of uninfected cells but not in cell nuclei treated with a proteasomal inhibitor or in cells microinjected with a pre-digested substrate. In infected cells, we observed that the green fluorescent degradation signal colocalizes with Hsc70 foci, our marker for VICE domains indicating that the 20S proteosome in VICE domains is active. Next we asked whether the reorganization of Hsc70, a major component of VICE domains, is essential for viral infection. We antagonized the activity of Hsc70 with a dominant-interfering form of Hsc70 that bears an inactivating single amino acid change in the ATPase domain (K71M) of Hsc70. Replication compartment formation is impaired in the presence of dominant-interfering Hsc70 suggesting that the activity of Hsc70 is required for efficient formation of replication compartments. We propose that during viral infection, a robust program of viral gene expression results in the production of large amounts of protein which overwhelms the capacity of the cell for folding. We hypothesize that the ability to form VICE domains may result in the sequestration of misfolded protein and the prevention of premature apoptosis.

Recognition of eight Rsp5 substrates by protein microarray

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Identifying enzyme substrates is one major focus of post-translational modification research and helps elucidate protein interaction networks and their complex regulation. We present a protein microarray-based in vitro method, which permits a systematic screening of immobilized proteins for their ubiquitylation by specific E3 ligases. Yeast Rsp5 is an essential HECT E3 ligase that is involved in several important biological processes. This high-throughput method allows the identification of Rsp5 substrates, which were supported by genetic interaction, and validated by in vitro and in vivo ubiquitylation experiments. Eight novel Rsp5 substrates were identified, and three among them are essential genes. The results corroborate with previous findings that Rsp5 is involved in proteasome function, DNA repair, chromosome segregation, assembly of actin, and transcription initiation. Further studies confirmed the effects of ubiquitylation on the function of the substrates. Protein microarray offers a powerful platform for in vitro screening of enzyme substrates and can be applied to any kind of post-translational modification experiments as well.

Adapter-mediated substrate selection in ER-associated degradation by a viral ubiquitin ligase

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An increasing number of viruses have been found to encode proteins that function in ubiquitin-mediated pathways. In many cases, the viral proteins serve to inhibit the host immune response and thereby enhance persistence and dissemination of the virus. One example of such a molecule is the mK3 protein of gamma-herpesvirus 68. This protein co-opts ER-associated degradation (ERAD) pathways to induce destruction of MHC class I molecules, causing their release from the ER for ubiquitin-dependent proteasomal degradation in the cytosol. The study of viral proteins like mK3 has yielded important insights into ERAD mechanisms. MK3 is an ER-resident RING-type (RING-CH) ubiquitin ligase (E3) that associates directly with the MHC class I peptide-loading complex (PLC) including TAP-1/2 and tapasin, and targets ubiquitination of nascent MHC class I heavy-chains. In the presence of mK3, class I heavy-chains, but not TAP-1/2 or tapasin, are detectably ubiquitinated and rapidly degraded. Therefore, this represents a unique system in which to study substrate selectivity in ERAD by E3 molecules. Previous work demonstrated that class I targeting by mK3 requires association between class I and TAP/tapasin, and a cytosolic tail on the class I heavy-chain. However, the specific tail sequence is not critical, as many diverse cytosolic tail sequences can support MHC class I degradation mediated by mK3. Here, we have extended these findings by conferring mK3 sensitivity upon a heterologous substrate, the human class I light-chain, beta$_2$-microglobulin (B_2m). Transmembrane and cytosolic domains of murine CD86 (B7.2) were appended to this normally soluble molecule to create TB_2m. TB_2m paired efficiently with class I heavy-chains and was surface-expressed, and rendered the class I heavy-chains sensitive to mK3 downregulation. Unlike TAP1/2 and tapasin, TB_2m itself was detectably ubiquitinated and rapidly degraded in the presence of mK3 in a tail-dependent manner. This is likely due to a favorable orientation of TB_2m in the PLC (compared with TAP-1/2 and tapasin) with respect to the RING-CH domain of mK3. These results support a model wherein TAP-1/2 and tapasin serve as adapter proteins and position mK3 in such a way that it can only ubiquitinate class I heavy chains (or TB2m when present). The recent appreciation that other viral and cellular RING-CH E3 molecules can target diverse substrates raises the possibility that adapter proteins facilitate substrate selection with these related E3 proteins, as well.

Adenovirus proteinase, a cysteine proteinase with deubiquitinating activity, slides rapidly along tens of thousands of base pairs of viral DNA via one-dimensional diffusion to locate its substrates

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The adenovirus proteinase (AVP) has been shown to contain a deubiquitinating activity. AVP shares a high degree of structural similarity to Ulp1, a cysteine proteinase that removes SUMO tags from proteins. We have been studying a different function of AVP, the role of AVP in the processing virion precursor proteins. However, our results may provide insight into how the deubiquitinating activity of AVP may locate its isopeptide substrates in the nucleus. Many viruses are assembled in part from precursor proteins which must be processed by a virus-coded proteinase before a nascent virus particle can become infectious, and this can present a conundrum. For example, during an adenovirus infection, how can 70 molecules of the adenovirus proteinase (AVP) cleave multiple copies of six different virion precursor proteins 3200 times in the core of a nascent virus particle where AVP and the virion precursor proteins are bound nonspecifically to the viral DNA? Either enzyme or substrate must move. However, being bound to the viral DNA, their rate of diffusion must be equal to the rate of diffusion of the DNA, and for a 36,000 base pair adenovirus genome packed into the virus particle at high pressure to a concentration of 500 g/L, that rate will be extremely small. The solution to this conundrum could be that AVP may locate the precursor proteins by reducing the dimensionality of its search from three- to one-dimension by sliding along the viral DNA via one-dimensional diffusion. This is the same search method utilized by nucleic acid binding proteins to find specific sites on DNA, e.g. RNA polymerase searching for a promoter. For proteins that do not consume biochemical energy to assist their diffusion, target search along DNA is a thermally activated (Brownian motion), directionally unbiased process. Total internal reflection fluorescence microscopy was used to observe directly and in real time the interaction of single, fluorescently-labeled molecules with flow-stretched lambda DNA with 3.5 ms time resolution and 11 nm spatial resolution. With AVP covalently linked to its 11-amino acid peptide cofactor pVIc, the proteinase exhibited directionless sliding on viral DNA that could last more than 1 sec and cover more than 20,000 base pairs. The one-dimensional diffusion constant for AVP-pVIc was the highest yet observed, 21×10^6 $(BP)^2/s$. This one-dimensional diffusion constant was not observed to increase when the salt concentration was increased ten-fold indicating that AVP-pVIc complexes predominantly slide in contact with DNA as opposed to hop on and off the DNA. The rapid sliding of fully-activated AVP-pVIc complexes on DNA is consistent with the model that the DNA inside adenovirus virions serves as a guide wire rapidly conducting the proteinase to its substrates, an unprecedented way for a proteinase to find its substrates and a new paradigm for virion maturation.

Possible role for ubiquitination in HHV-7-mediated immune evasion

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The viral immunoevasin U21 from human herpesvirus-7 binds to and directs class I MHC molecules to the lysosomal compartment. U21, a type I integral membrane protein, associates with properly-folded class I MHC molecules soon after synthesis, in the ER, and the two molecules travel together to the lysosomal compartment. Because lysosomal sorting signals are often located within the cytoplasmic tails of membrane proteins, we originally hypothesized that U21 might mediate the diversion of class I molecules through a lysosomal sorting signal contained within its cytoplasmic tail, much like the m06 (gp48) immunoevasin from MCMV. Surprisingly, the cytoplasmic tail of U21 is not necessary for its ability to divert class I MHC molecules, suggesting a novel mechanism for U21-mediated lysosomal sorting. Here we present evidence that the cytoplasmic tail of class I MHC molecules may be modified on lysine residues as a result of U21 expression. These modifications may lead to relocalization of both proteins to the lysosomal compartment.

A novel role of Ubc9 in antitumor immunity: High expression of Ubc9 in melanoma cells may exert an anti-Inflammatory role and prevent migration of immune cells at the tumor site

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We have recently shown that Ubc9, the only SUMO E2 conjugating enzyme, is highly expressed in melanoma-infiltrated lymph nodes and that its downregulation *in vitro* suppresses melanoma cell proliferation and enhances the cytotoxic effect of chemotherapeutic agents (*Moschos et al. Oncogene 2007*). To assess whether high expression of Ubc9 in melanoma promotes cancer growth via other mechanisms we performed a series of studies characterizing the role of Ubc9 in relation to host antitumor immunity. Ubc9 protein expression in metastatic melanoma cell lines was suppressed with SmartPool Ubc9 vs. RISC-Free negative control small interfering RNA (siRNA). xMAP multiplex immunobead assay technology (Luminex Corp., Austin, TX) was used to simultaneously test 29 cytokines, chemokines, angiogenic as well as growth factors in the supernatant of melanoma cells 120 hours after siRNA transfection. Immunoblot analysis and luciferase promoter assay was used to study the effect of Ubc9 suppression upon several signal transduction molecules. Flow cytometric analysis was used to assess the effect of Ubc9 suppression: a) in melanoma surface markers associated with antigen presentation and inflammation; b) in allogeneic peripheral blood mononuclear cell (PBMC) function after coculture in the presence or absence of stimulation with OKT3 and IL-2. Chromium release assay was used to study whether Ubc9 suppression in melanoma cells made them more prone to cytotoxic cell death. Suppression of Ubc9 in melanoma cells increased expression of both total and activated STAT1 (Tyr701) and increased activity but not actual levels of nuclear factor kappa B. Melanoma cells in which Ubc9 was suppressed expressed higher levels of the antigen presenting molecule HLA-ABC and the pro-inflammatory molecule FasL and they secreted higher levels of the pro-inflammatory *h*igh *m*obility *g*roup *b*ox 1 (HMGB1) protein as well as the pro-inflammatory cytokines IL-6, IL-8, MCP-1, RANTES, and IP-10. Suppression of Ubc9 in melanoma cell lines did not enhance either cell mediated cytotoxicity or proliferation of cocultured allogeneic PBMCs although it increased the number of PBMCs bound to melanoma cell lines. High expression of Ubc9 in melanoma cells may have an anti-inflammatory role, suppressing antigen presentation and secretion of pro-inflammatory cytokines. This tumor escape mechanism may be another oncogenic role of Ubc9 in melanoma, apart from suppression of apoptosis and promotion of cell proliferation. Studies are ongoing to assess the clinical importance of Ubc9 overexpression in melanoma and whether other aspects of PBMC function, such as cytotoxicity, activation, maturation, or survival are affected.

Proteasome inhibitors have detrimental effect on replication of vesicular stomatitis virus

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The replication of viruses depends on the conditions of the infected cells and may be affected by the stresses induced in the cells by drugs. It was previously shown that the inhibition of proteasomes at the late stages of vesicular stomatitis virus (VSV) replication prevented the budding of viruses from infected cells without any significant inhibition of virus protein and RNA synthesis. In this study, we examined the effect of proteasome inhibitors on the early steps of VSV replication. We found that the inhibitors of proteasomes significantly suppressed VSV protein synthesis, virus accumulation, and protected infected cells from toxic effect of VSV replication. The detrimental effect on virus replication was detected when the inhibitors of proteasomes have been administrated during first four hours of infection. We demonstrated that the inhibition of proteasome activity in combination with VSV infection induced the stress in the cells. This stress caused the suppression of translation of cellular and viral mRNA molecules. Although VSV infection and the inhibition of proteasomes alone had the detrimental effects on cellular translation process, their combination inhibited translation even stronger. Thus, proteasome inhibition may represent a novel therapeutic approach against virus infection.

A role for INF-α/β and ISG15 in inhibiting ebola virus budding

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Ebola virus budding is mediated by overlapping L-domains within the VP40 matrix protein. The L domains (PTAP and PPEY) of VP40 are known to mediate interactions with Tsg101 and the ubiquitin ligase, Nedd4. Ubiquitin is thought to be exploited by HIV-1 and Ebola virus, and these virus-host interactions facilitate efficient virus budding. ISG15 is a ubiquitin-like protein whose expression is induced by type-1 IFN. Over-expression of ISG15 has been shown to disrupt HIV-1 L-domain-host interactions and inhibit virus budding. We found that over-expression of ISG15 inhibited budding of Ebola virus VP40 VLPs. Indeed, co-expression of ISG15 and ISG15 conjugating enzymes, UbE1L and Ubc-8, completely blocked budding of VP40 VLPs. Importantly, ISG15 expression did not inhibit synthesis of VP40 in cell extracts. ISG15 over-expression also inhibited ubiquitin conjugation of VP40 VLPs. Lastly, IFN-induced ISG15 also inhibited release of VP40 VLPs. Interestingly, the budding defective mutant of VP40, ΔPTPY, was not affected by over-expression of ISG15. These data provide evidence that the innate antiviral response may target late steps in budding of Ebola virus. A better understanding of this process may provide the basis for the development of novel therapeutics to block virus release.

The role of ubiquitin and ubiquitin-like molecules in neuronal persistence of botulinum neurotoxin

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Botulinum neurotoxin (BoNT) exposure of certain serotypes is characterized by extreme persistence of the flaccid paralysis. The enduring clinical symptoms of intoxication are particularly prominent with BoNT serotype A (BoNT/A) and are caused by retention of the toxin protease light chain (LC) in the presynaptic terminal of the neuromuscular junction. Three BoNT serotypes cleave SNAP25 but differ greatly in persistence of activity. BoNT/A and /C are similarly long (weeks) while BoNT/E is quite short (hours). We are investigating BoNT LC trafficking, post-translational modifications, and cellular protein interactions to determine the mechanism of differences in persistence. Trafficking of BoNT/A LC follows a pathway from the endosome and MVB to the plasma membrane. We are investigating the role of ubiquitin and ubiquitin-like modifications and deubiquitination in regulating this trafficking. We are also developing therapeutic agents that accelerate proteasome-mediated turnover of BoNT/A. We find that the UPS degrades BoNT/E much more rapidly than BoNT/A LC in transfected N18 cells suggesting the differential rates of proteasome degradation may account for the disparity in neuronal persistence. We are investigating whether preferential ubiquitination of BoNT/E or protective deubiquitination of BoNT/A are factors in the differential turnover of these proteins. Cell pull-down assays and yeast two-hybrid analysis of BoNT/A and /E LC interactors has revealed a number of “hits” that are related to ubiquitin and deubiquitination. Early results suggest that BoNT/A LC may recruit deubiquitinating enzymes. We have produced a family of “designer” E3 ubiquitin ligases designed to specifically attach ubiquitin to the BoNT/A LC, thus designating it for destruction by the cellular UPS. This approach to development of designer E3 ubiquitin ligases is based on the knowledge that most cellular E3 ubiquitin ligases are modular in structure. One E3 module binds to the target protein (substrate) and a second module promotes ligation of ubiquitin to the substrate protein. To generate BoNT/A LC-directed designer ubiquitin ligases, we utilized a number of E3 catalytic domains (F-box, RING, HECT, R-IBR-R, U-box) and degron sequences fused to A-LC-directed targeting domains. One A-LC binding domain is a non-cleavable form of SNAP25 (SNAP25nc) while the second targeting domain is a high affinity recombinant anti-BoNT/A LC single-chain camelid antibody (VHH) designated B8. Testing of these agents in transfected neuroblastoma cells indicates that such designer ubiquitin ligases can be effective in accelerating BoNT/A LC degradation. The B8 VHH with affinity in the low nM range is the more effective targeting domain and the F-box domain appears to be the most effective ligase domain. We are pursuing protein delivery systems for the B8 VHH-F box designer ubiquitin ligases.

Adeno-associated viruses can induce phosphorylation of eIF2a via PKR activation which can be overcome by helper Ad5 VA RNA

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We have recently shown that the Ad5 E4Orf6 protein together with E1B-55K, can target adeno-associated virus type 5 (AAV5) capsid and small Rep proteins for degradation via the proteasome, and at least part of the required help that VA I RNA provides during AAV5 infection is to increase accumulated levels of viral proteins necessary for replication. We have now found that mutants of adenovirus type 5 (Ad5) VA I RNA deficient in inhibiting the activation and subsequent phosphorylation of PKR could neither function as helpers for AAV5 replication, nor enhance AAV5 protein accumulation in either the presence or absence of Ad5 E4Orf6 and E2a. Furthermore, a short region of the AAV5 capsid gene RNA leader sequence surrounding the AUG of VP1 could induce the phosphorylation of eIF2a. Both siRNA directed against PKR, and the addition of the herpes simplex virus ICP34.5 protein, enhanced the accumulation of AAV5 capsid protein in the presence of the AAV5 capsid-gene PKR-inducing element, suggesting that VA RNA acted to overcome direct AAV5 induced activation of PKR that led to phosphorylation of eIF2a. Expression of both the closely related Go-AAV and the prototype AAV2 capsid gene transcription units also induced the phosphorylation of eIF2a suggesting that induction of the PKR/eIF2a cellular response may be a previously unrecognized general feature of at least the *Dependovirus* genera of the *Parvovirinae*.

HIV-1 accessory proteins VPR and Vif inhibit antiviral response by targeting IRF-3 for degradation

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The activation of IRF-3 during the early stages of viral infection is critical for the initiation of the antiviral response; however the activation of IRF-3 in HIV-1 infected cells has not yet been characterized. We demonstrate that the early steps of HIV-1 infection do not lead to the activation and nuclear translocation of IRF-3; instead, the relative levels of IRF-3 protein are decreased due to the ubiquitin associated proteosome degradation. Addressing the molecular mechanism of this effect we show that the degradation is independent of HIV-1 replication and that virion associated accessory proteins, Vif and Vpr can independently degrade IRF-3. The null mutation of both of these two genes reduced the capacity of the HIV-1 virus to down modulate IRF-3 levels. The degradation was associated with Vif and Vpr mediated ubiquitination of IRF-3 and was independent of the activation of IRF-3. Mutanttion analysis have shown that N-terminal lysine residues play a critical role in the Vif- and Vpr-mediated degradation of IRF-3. These data implicate Vif and Vpr in the disruption of the initial antiviral response and point to the need of HIV-1 to circumvent the antiviral response during the very early phase of replication.

Vpr and Vif manipulate the cell cycle through recruitment of distinct E3 ubiquitin ligases

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The Vpr proteins encoded by HIV-1, HIV-2, SIVmac, and SIVagm have the distinct capacity to induce cell cycle arrest at the G_2/M boundary. G_2 arrest by these viral proteins leads to transactivation of the viral promoter and, as a late event, induction of apoptosis in infected cells. Recently, we and others have demonstrated that, for HIV-1 Vpr, the above functions are effected through the recruitment and activation of a cullin 4/DDB1/DCAF1 E3 ubiquitin ligase. We have also observed that the the Vpr homologs from HIV-2, SIVmac and SIVagm, which also induce G_2 arrest, recruit the cullin 4/DDB1/DCAF1 E3 ligase complex, despite the limited amino acid sequence conservation among them. Interestingly, the Vpx homolog, which is encoded by HIV-2 and SIVmac, does not induce cell cycle alteration, but still targets the cullin 4/DDB1/DCAF1 ligase.

HIV-1 Vif targets APOBEC3G for ubiquitination, via recruitment of a cullin 5-based E3 ligase complex. We have recently observed that Vif can also induce activation of the G_2 checkpoint. Thus, given the similarities between Vpr and Vif, we have undertaken mechanistic studies to better understand how these functions of Vif are inter-related. We find that Vif's ability to induce G_2 arrest is independent of the presence or absence of APOBEC3G, but is still dependent on the presence of a functional ubiquitin/proteasome system. Furthermore, site directed mutagenesis of the Vif regions that mediate interaction with either APOBEC3G or cullin 5 ablated Vif-mediated induction of G_2 arrest. Therefore, it is tempting to speculate that (a) Vif targets a checkpoint protein that is different from APOBEC family proteins; and (b) this target may be related or identical to the degradation target of Vpr.

Crystal structure of the Hse1:Vps27 core: A protein complex mimicked by viral matrix proteins

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Budding of retroviruses, rhabdoviruses, and filoviruses is mediated by ubiquitylation of their matrix protein and by a PTAP-like motif that they carry. While the ubiquitylation signal mimics normal transmembranal cargo entry to the ESCRT machinery, the PTAP-like sequence competes with the endogenous interaction of Hrs:STAM complex with ESCRT-I. Understanding the endogenous process of sorting via the ESCRT system is therefore critical for our understanding of how viruses use the hijacked system for budding, and may serve as target for drug design. The yeast Vps27:Hse1 complex is structurally and functionally conserved with the mammalian Hrs:STAM complex. The Vps27:Hse1 complex directly binds to ubiquitinated transmembrane proteins and recruits both ubiquitin ligases and deubiquitinating enzymes. We have solved the crystal structure of the core responsible for the assembly of the Vps27:Hse1 complex at 3.0Å resolution. The structure consists of two intertwined GAT domains, each consisting of two helices from one subunit and one from the other. The two GAT domains are connected by an antiparallel coiled-coil to form a 90 Å-long barbell-like structure. This structure places the domains of Vps27 and Hse1 that recruit ubiquitinated cargo and deubiquitinating enzymes close to each other. Coarse-grained Monte Carlo simulations of the Vps27:Hse1 complex on a membrane show how the complex binds cooperatively to lipids and ubiquitinated membrane proteins and acts as a scaffold for ubiquitination reactions.

Investigating the mechanism of regulation of HIV-1 biogenesis by the E3 ligase POSH

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We have conducted a rational siRNA screen for the identification of E3 ligases involved in HIV-1 late phase. The screen identified three Ring finger E3 ubiquitin ligases, the silencing of which had significant effect on virus production: Cbl-b, a novel gene internally termed PRT14-2, and Plenty of SH3, POSH a protein initially identified as a scaffold for the JNK pathway kinases. We have subsequently shown that POSH-mediated ubiquitination controls virus production through a mechanism that involves regulation of intracellular trafficking at the trans Golgi network [Alroy et al. (2005) *Proc. Natl. Acad. Sc. USA* ***102***, 1478]. To further understand the role of POSH in virus biogenesis we set out to identify POSH ubiquitination substrates. The study identified two substrates. The first identified substrate is the ubiquitin domain Homocysteine-inducible Endoplasmic Reticulum Protein, Herp. Herp serves as a substrate as well as an activator for POSH-mediated K63-linked polyubiquitination. Ubiquitination of the Ubl domain of Herp by POSH is required for the redistribution of Herp from the TGN to the ER upon calcium perturbation. Consistent with the established role of Herp in restriction of intracellular calcium, POSH activity is also required for attenuation of calcium burst when ER calcium ATPase is inhibited [Tuvia et al. (2007) *J. cell Biol.* ***177***, 51]. Since HIV-1 release is also significantly inhibited in Herp depleted cells we speculate that regulation of calcium homeostasis by POSH during HIV-1 infection is important for HIV-1 biogenesis. The second identified POSH ubiquitination substrate is Alix. Alix, an ESCRT-III associated protein binds to YPX_nL late domain motif. The role of Alix/AIP1 in HIV-1 budding and potentially in the biogenesis of other viruses was recently shown [Fisher et al. (2007) *Cell* ***128***, 841; Lee et al. (2007) *Nat. Struct. Mol. Biol.* ***14***, 194]. We are currently investigating whether POSH-mediated ubiquitination of Alix is essential for the function of Alix in the production of HIV-1 and possibly in the biogenesis of other viruses.

Expression of FAT10 and its Covalent Conjugates in Healthy and Diseased Tissues

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FAT10 is a ubiquitin-like protein (UBL) that is upregulated in renal tubular epithelial cells (RTEC) in HIV-associated nephropathy (HIVAN) and is an important mediator of HIV-induced RTEC apoptosis. In addition to its role in apoptosis, FAT10 influences immune response and cell cycle regulation. The ability of UBLs to alter the function of target proteins is mediated primarily via covalent conjugation of UBLs to target proteins. We therefore studied expression of FAT10 protein and covalent FAT10 conjugates in kidneys from HIV-transgenic mice and in immune tissue including thymus, lymph nodes, and spleen. Since tubulointerstitial inflammation and apoptosis of renal cells are important pathogenic factors in many renal diseases, we examined FAT10 expression in biopsy specimens of diabetic nephropathy (DN), hypertensive nephrosclerosis (HN), and IgA nephropathy (IgAN). To determine the expression of FAT10 and FAT10 covalent conjugates in tissue, we harvested kidneys and immune organs (thymus, lymph nodes, and spleen) from normal mice and kidneys from HIV- transgenic mice. Protein lysates were subjected to SDS-PAGE prior to western blotting with anti- FAT10. To study FAT10 expression in common renal diseases, we performed immunostaining using anti-FAT10 on renal biopsies from patients with DN, HN, and IgAN (5 patients with each disease). FAT10 was strongly expressed in kidneys from HIV-transgenic mice and thymus, lymph nodes, and spleens from normal mice. Interestingly, the majority of FAT10 detected in kidneys, thymus, and spleen from normal mice was monomeric. However, most FAT10 in normal lymph nodes and kidneys from HIV-transgenic mice was in the form of high molecular weight covalent conjugates. FAT10 was highly expressed in RTEC of patients with DN, HN, and IgAN. In conclusion, FAT10 is expressed in normal immune tissue and is upregulated in RTEC in several renal diseases. Further studies investigating the mechanism of covalent modification of proteins by FAT10 will improve our understanding of the role of this UBL in disease pathogenesis.

ICP27 interacts with the C-terminal domain of RNA polymerase II and facilitates its recruitment to herpes simplex Virus-1 transcription sites, where it undergoes ubiquitination and proteasomal degradation during infection

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Herpes simplex virus 1 (HSV-1) ICP27 has been shown to interact with RNA polymerase II (RNAP II) holoenzyme. We have shown that ICP27 interacts with the C-terminal domain (CTD) of RNAP II and ICP27 mutants that cannot interact fail to relocalize RNAP II to viral transcription sites, suggesting a role for ICP27 in RNAP II recruitment. Using monoclonal antibodies specific for different phosphorylated forms of RNAP II CTD, we found that the serine-2 phosphorylated form, which is found predominantly in elongating complexes was not recruited to viral transcription sites. Further, there was an overall reduction in phosphoserine-2 staining. Western blot analysis revealed that there was a pronounced decrease in the phosphoserine-2 form and in overall RNAP II levels in lysates from cells infected with wild type HSV-1. Treatment of infected cells with proteasome inhibitors MG-132 and lactacystin prevented the decrease in the phosphoserine-2 form and in overall RNAP II levels, suggesting that protein degradation was occurring. Interestingly, there was a concomitant decrease in the levels of several HSV-1 late proteins and in virus yield. Proteasomal degradation has been shown to resolve stalled RNAP II complexes at sites of DNA damage to allow 3' processing of transcripts. Thus, we propose that at later times of infection when robust transcription and DNA replication are occurring, elongating complexes may collide and proteasomal degradation may be required for resolution. Further support for this hypothesis comes from ChIP assays on HSV-1 late transcripts, which demonstrated that ubiquitin was associated with the coding regions of HSV-1 late genes during infection. In addition, it has been shown that the cellular chaperone protein Hsc70, along with components of the 26S proteasome and ubiquitin-conjugated proteins become sequestered in discrete foci in the nuclei of HSV-1 infected cells (A.D. Burch and S.K. Weller, J. Virol. 78:7175-7185, 2004). We have found that ICP27 also interacts with Hsc70, and is required for the formation of Hsc70 nuclear foci. During infection with ICP27 mutants that are unable to recruit RNAP II to viral replication sites, viral transcript levels were greatly reduced and Hsc70 focus formation was also curtailed. Further, a dominant negative Hsc70 mutant that cannot hydrolyze ATP, partially rescued RNAP II degradation during HSV-1 infection, and an increase in ubiquitinated forms of RNAP II was observed, indicative of some stabilization. There was also a decrease in virus yields, suggesting that proteasomal degradation of stalled RNAP II complexes during robust HSV-1 transcription benefits viral gene expression. We propose that Hsc70 foci serve to facilitate this process.

Ubiquitination of β-Arrestin Links 7-Transmembrane Receptor Endocytosis and ERK Activation

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β-arrestin2 is a multifunctional protein that regulates trafficking and signaling of many cell surface receptors and plays crucial roles in cell growth, apoptosis and modulation of immune functions. β-arrestin is also ubiquitinated upon 7 transmembrane receptor (7TMR) activation and this process is required to mediate rapid receptor endocytosis. To better understand the connection between ubiquitination and β-arrestin's endocytic and signaling functions, we generated a β-arrestin2 mutant that is defective in ubiquitination (β-arrestin2^{0K}), by mutating all the ubiquitin acceptor lysines to arginines and compared its properties with the wild type and a stably ubiquitinated β-arrestin2-Ub chimera. In cellular coimmunoprecipitation assays, β-arrestin2^{0K} bound non-receptor partners, such as AP2 and c-Raf and scaffolded pERK as robustly as the wild type β-arrestin2 but displayed weak binding to clathrin. Moreover, β-arrestin2^{0K} was recruited only transiently to activated receptors at the membrane, did not enhance receptor internalization and decreased the amount of pERK assimilated into isolated β2 adrenergic receptor (β2AR) complexes. In comparison, wild type β-arrestin2 formed ERK signaling complexes with the β2AR at the membrane while a stably ubiquitinated β-arrestin2-Ub chimera, not only stabilized the ERK signalosomes but also led to their endosomal targeting. Interestingly, in cellular fractionation assays the ubiquitination state of β-arrestin2 favors its distribution in membrane fractions suggesting that ubiquitination increases β-arrestin's propensity for membrane association. Our findings suggest that although β-arrestin ubiquitination is dispensable for β-arrestin's cytosol to membrane translocation and its 'constitutive' interactions with some cytosolic proteins, it nevertheless is a prerequisite both for the formation of tight complexes with 7TMRs *in vivo* as well as for membrane compartment interactions that are crucial for downstream endocytic and signaling processes.

Ubiquitination of the inactive foot-and-mouth disease virus in dendritic cells

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Foot-and-mouth disease (FMD) caused by the foot-and-mouth disease virus (FMDV) is one of the most highly contagious diseases of animals and humans.
Dendritic cells (DCs) are professional antigen-presenting cells (APCs) with the unique property of inducing priming and differentiation of naive CD4+ and CD8+ T cells into helper T cells and cytotoxic effectors. It is known that the majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway (UPP), and the UPP is one of the pathways of antigen processing and presentation in DC. In the UPP, proteins targeted for proteasomal degradation are first ligated to multiple ubiquitin molecules. However, the mechanisms underlying the presentation of the FMDV antigen are poorly understood. To address this issue, we pulsed monocyte-derived DCs of Balb/c mice with inactive FMDV in vitro to determine if ubiquitination is involved in the processing of the FMDV antigens.
The cell lysates of FMDV-pulsed DCs were subjected to SDS-PAGE and immunoblotted with two anti-ubiquitin antibodies(clone FK1 and clone FK2). It was clearly shown that the ubiquitinated FMDV protein of approximately 75kD could be detected at 0.5h, 3h, 6h, 10h, and 20h immediately after FMDV- loading onto DCs. Our data show that the ubiquitinated protein may be VP1 in terms of calculation of the molecular weights of VP1 and the ubiquitin chain. Moreover, monoclonal antibody of clone FK1 recognises only polyubiquitinylated proteins which were degradated by the 26S proteasome in most cases, whilst clone FK2 recognises both mono-and poly-ubiquitinylated species and the monoubiquitination facilitate the degradation by the lysosome pathway. In the study, the levels of ubiquitinated proteins recognized by FK2 were greater than those by FK1, indicating the lysosome play a major role in the processing of the FMDV antigens.
Taken together, these findings show for the first time that the protein antigen of inactive FMDV are recognized by the ubiquitinating system and targeted to the lysosome for processing in DCs, implying a cross-processing route of ubiquitinated FMDV protein antigens.

Identification of membrane trafficking factors involved in hepatitis C virus infection

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Globally, approximately 170 million people have been infected with hepatitis C virus (HCV). HCV infection can result in severe liver disease that includes chronic hepatitis, cirrhosis and hepatocellular carcinoma. The current therapy, pegylated interferon in combination with ribavirin, is expensive, ineffective in many HCV patients and associated with significant side effects. The cell biology of HCV is understudied and the specific cellular pathways and host factors involved in viral replication are largely unknown. We initiated an RNAi based screen to identify host determinants required in HCV infection. 140 different siRNAs targeting various cellular trafficking pathways were analyzed for effects on HCV RNA replication and infectious virus production. Our first-pass screen identified several interesting host factors that appear to play important roles in the HCV lifecycle, including factors previously reported to modulate ubiquitin modification pathways. We are currently using various approaches to confirm and dissect their mechanism of action in HCV-infected cells.

Functional analysis of the RAG1 V(D)J recombinase protein's ubiquitin ligase activity

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The RAG1 immune-specific recombinase protein is required for V(D)J recombination, a programmed DNA rearrangement crucial to the development of the vertebrate immune system. The zinc-binding C_3HC_4 RING domain of RAG1 functions as a ubiquitin ligase (E3). Although the RING finger domain is not required for RAG1 DNA cleavage activity, amino acid substitutions and deletions in this region influence both V(D)J recombination in model systems and immune development in human patients, suggesting a critical role for RAG1 E3 activity. We examined the effect of amino acid substitutions in the RAG1 RING finger domain on its functional interaction with ubiquitin conjugating (E2) enzymes. A rational mutagenesis approach was taken in which the RAG1 RING domain crystal structure was aligned *in silico* with the crystal structure of the cbl RING domain bound to the E2 enzyme UbcH7 in order to predict the RAG1-E2 interface. The majority of RAG1 single and double amino acid substitutions selected based on this approach did not disrupt the ability of the purified RAG1[218-389] fragment to functionally interact with the E2 enzyme CDC34 in our reconstituted ubiquitylation assay. This was true even for substitutions known to disrupt functional interaction for other E2-E3 pairs. In addition, none of the variant RAG1 proteins was able to promote ubiquitylation in the presence of an E2 enzyme other than CDC34, indicating that we had not loosened the apparent specificity of RAG1 for CDC34. As expected, individual substitutions at the zinc coordinating residues C325 and C328 (C325G, C325Y and C328S) severely abrogated RAG1 E3 activity, as did substitution at the P326 position (P326G) where a bend in the polypeptide backbone is required. Work in our lab and elsewhere confirms that all of these substitutions show some defect in V(D)J recombination in cultured cells, although to differing extents. The C325Y and P326G mutant proteins were further analyzed for alterations in their tertiary structures. Partial proteolysis generated a trypsin-resistant fragment spanning amino acids 254-377 for both the wild type and the P326G proteins, but not for the C325Y. These data indicate that the C325Y substitution disrupts the tertiary structure of the RING domain, but that the overall fold can be maintained with the P326G substitution. The inability of P326G to functionally interact with CDC34 may be due to a specific role for P326 in the E2-E3 interface.

Inhibition of the anaphase-promoting complex during human cytomegalovirus infection is associated with the hyperphosphorylation of Cdh1 and the dissociation of the APC1 subunit

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Human cytomegalovirus (HCMV) replication is temporally regulated and involves an intricate set of interactions between the virus and the host cell machinery that optimize the cellular environment for viral replication and assembly. HCMV induces cells towards S phase such that the host's DNA synthesis machinery is activated and available for viral DNA replication. However, subsequent dysregulation of multiple cellular factors involved in the cell cycle inhibits host cell DNA synthesis and causes the cell to arrest in a pseudo-G1 state that is distinguished by the expression of selected S-phase and M-phase gene products. The cell cycle arrest upon HCMV infection is due in part to the inactivation of the Anaphase-Promoting Complex (APC), an E3 ubiquitin ligase that mediates the timely degradation of various cell cycle regulators. APC activity is governed by several factors, including activation by either one of two co-activators, Cdc20 and Cdh1. We have elucidated the mechanism by which HCMV-induced inactivation of the APC occurs. Our results show that Cdh1 becomes significantly upregulated by 8–12 h post infection, and accumulates in a hyperphosphorylated form that likely prevents its association and activation of the APC. The hyperphosphorylation does not appear to be CDK-dependent, as it was not affected upon treatment with the CDK inhibitor Roscovitine. However, in vitro binding assays suggest that Cdh1 modification alone is not sufficient to account for its lack of association with the APC in HCMV-infected cells. We further show that the core APC also becomes destabilized at this time as the largest subunit, APC1, dissociates from the complex. Dissociation of the core complex itself likely accounts for the observed inactivity and inhibits its interaction with Cdh1. Although APC activity is inhibited very early during the infection, our results indicate that viral input alone is not sufficient to account for the observed inactivation and that viral early gene expression is likely required. Taken together, HCMV appears to have adopted multiple mechanisms to inactivate the APC, which underscores its importance for a productive infection.

DDB1 and proteasomal activity are required for HIV-1 Vpr-induced G2 arrest

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Vpr-mediated induction of G2 cell cycle arrest has been postulated to be important for HIV-1 replication, but the precise role of Vpr in this cell cycle arrest is unclear. In the present study, we have shown that HIV-1 Vpr interacts with damaged DNA binding protein 1 (DDB1) but not its partner DDB2. However, Vpr binding to DDB1 was not sufficient to induce G2 arrest. A reduction in DDB1 expression in the absence of Vpr also did not induce G2 arrest. On the other hand, Vpr-induced G2 arrest was impaired when the intracellular level of DDB1 was reduced by siRNA treatment. Furthermore, Vpr-induced G2 arrest was largely abolished by a proteasome inhibitor. These data suggest that Vpr assembles with DDB1 to form an E3 ubiquitin ligase that targets cellular substrates for proteasome-mediated degradation and G2 arrest.

Broad antiviral effects by members of the TRIM family of E3 ligases

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TRIM proteins describe a family of E3 ligases that share a characteristic N-terminal tripartite domain structure containing Ring, B box and coiled coil domains. While a number of TRIM family members have been reported to exhibit antiviral activity, a systematic analysis has not been performed. Because many of them are not ubiquitously expressed or are induced by interferons we decided use a transient expression based screen in HEK293 cells to identify antiretroviral TRIM proteins. Out of 54 TRIM proteins (35 human and 19 mouse) analyzed, 21 exhibited potent antiviral activities against several retroviruses including the human immunodeficiency virus 1(HIV-1), murine leukemia virus (MLV), and avian leukosis virus (ALV). We confirm the previously identified inhibitory effects of TRIM 1 and 5 on viral entry and also identify two new endogenous TRIM proteins whose silencing leads to 3-fold enhancement in HIV entry. We also identify another TRIM protein that interferes with the endogenous Ref1 restriction by degrading TRIM5 alpha in a proteasome dependent manner. Interestingly, multiple TRIM proteins exhibit potent antiviral effects affecting the late stages of the viral life cycle. Some TRIM proteins down-regulate expression from retroviral LTR promoters while others interfere with retroviral budding and egress. Silencing of a few interfering TRIM proteins led to an enhancement or inhibition of viral egress. Thus in addition to being bonafide restriction factors our screen revealed that TRIM proteins also play an essential role in retroviral replication. Because the majority of the TRIM proteins exhibiting antiviral activities are up-regulated in response to interferons, these data suggest that they contribute to the restriction of retroviral infection in immunoprivileged cells.

Display of PEST motifs on the surface of bacteriophage lambda lead to improved gene transfer efficiency in mammalian cells

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Bacteriophage lambda can be used as a vector to express exogenous genes in mammalian cells and tissues. Optimizing the efficacy of gene delivery by this vector may lead to an alternate class of gene transfer vectors that may be used alone or in concert with other vector modalities. In our efforts to increase the efficiency of gene delivery by bacteriophage lambda, PEST-like motifs were displayed on the surface of lambda through fusion with the major capsid protein (gpD). We reasoned that PEST motifs might enhance phage uncoating in mammalian cells, by targeting phage to the proteasome. Gene transfer efficiency by the PEST displaying phage particles was measured by quantitating the expression of a CMV promoter-driven luciferase reporter gene encoded in the phage genome. *In vivo* gene expression analysis in mice revealed that two PEST motifs improved gene transfer *in vivo*. We sought to delineate the mechanism of action of these PEST motifs, by performing *in vitro* studies in 293 cells and COS cells, using luciferase expression as a measure of gene transfer efficiency. The PEST-bearing phage particles were more efficient at mediating gene transfer *in vitro*, when compared to unmodified phage. Experiments were then performed in which cells were incubated with phage particles in the presence or absence of drugs that inhibit proteasome function (lactacystin) or endosome acidification (chloroquine). Unexpectedly, both drugs enhanced gene expression. This suggests that PEST motifs do not enhance phage-mediated gene transfer because they target phage to the proteasome for more efficient uncoating. Rather, the proteasome appears to be a site for non-productive degradation of phage - as suggested by the increase in phage-mediated gene transfer in the presence of lactacystin. The results with chloroquine suggest that, *in vitro* at least, phage particles can enter mammalian cells through an endosome-mediated pathway. These results further our understanding of phage-mediated gene transfer in mammalian cells and suggest approaches to increase the efficiency of gene delivery by lambda phage vectors.

The human papilloma virus type-16 E6 oncoprotein interacts with the cellular de-ubiquitinating enzyme USP15

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Human papilloma virus (HPV) is associated with greater than 90-95% of cervical cancers. HPV encodes two oncoproteins whose combined efforts drive the transformation of epithelial cells. The E7 oncoprotein complexes with the tumor suppressor protein Rb and targets it for ubiquitin-mediated degradation. The release of E2F from Rb leads to cell cycle entry. The E6 oncoprotein exerts its effects via complexing with the ubiquitin E3 ligase E6AP and the tumor suppressor protein p53 to target the ubiquitin-mediated degradation of p53. Suppression of p53 inhibits the apoptosis that results from aberrant activation of E2F by E7. E6AP also mediates the degradation of several other E6 partner proteins, as well as E6 itself. Here we report the first evidence of HPV16E6 interacting with a deubiquitinating enzyme, the ubiquitin-specific protease USP15. Over-expression of USP15 results in increased steady state levels of E6. Half-life analysis indicates that USP15 acts through stabilization of E6 at the protein level. The catalytically active site of USP15 is required for E6 stabilization. E6 frequently targets its binding partners for degradation as mentioned above, however we were unable to detect E6 mediated degradation of USP15. These data suggests that E6, and possibly E6AP in complex with E6, may be a target for USP15 deubiquitylation activity. Current experiments are focused on studying the effect of USP15 knock down on E6 expression/stability in various cell lines.

PLIC-1 inhibits the TLR-mediated antiviral pathway

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Toll-like receptors (TLRs) are key innate immune receptors that recognize non-self pathogens and hence trigger host responses. We have recently identified an ubiquitin-like protein named protein linking integrin associated protein to cytoskeleton 1 (PLIC-1) that interacts with the cytoplasmic domain of TLR4. The interaction between TLR4 and PLIC-1 was verified by co-immunoprecipitation. Further studies suggest that PLIC-1 may be interacting with multiple TIR domain containing proteins. PLIC-1 decreased the production of TRIF in an overexpression system. Consequently, PLIC-1 was found to inhibit the TRIF-mediated activation of both NF-kappaB and IFN-beta pathways in macrophages. Consistently, reduction of endogenous PLIC-1 by shRNAi delivered via retroviral infection enhanced the cellular responses to LPS or poly I:C. Lastly, the inhibitory role of PLIC-1 in regulating TLR-mediated antiviral responses was confirmed in several viral infections. Together, our results have implicated PLIC-1 as a negative regulator of TLR-mediated antiviral pathway. This work is supported by the University of Pittsburgh faculty start-up fund.

Viral regulation of the PKR innate response by specific proteolytic targeting

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Viruses have evolved a multitude of mechanisms to counteract host innate defenses. The double-stranded RNA-dependent protein kinase, PKR, is involved in many of these host defense processes including translational regulation and signal transduction activation. The importance of this enzyme as a key player in the inhibition of viral replication makes it a common target for viral countermeasures. An emerging theme to many viruses involves targeting PKR for proteolytic degradation. The ranavirus, ATV, encodes a pseudosubstrate for PKR which binds to and specifically targets PKR for rapid ubiquitination and proteasomal degradation. Mouse hepatitis virus (MHV) and encephalomyocarditis virus (EMCV) both induce proteolytic degradation of PKR, however this appears to not require ubiquitination of PKR. Degradation of PKR by all these viruses likely requires PKR binding to dsRNA which may expose a PEST domain located within the PKR sequence. The degradation of PKR during MHV and EMCV infection is blocked following interferon treatment. For EMCV, this interferon-induced block in PKR degradation requires the presence of the ubiquitin-like protein, ISG15.

Specificity of cellular and viral deISGylating enzymes

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Recently, a variety of viral processing proteases have been shown to possess deubiquitinating and/or deISGylating enzyme activity, That is, they are able to reverse to post-translational modification of proteins by the removal of the ubiquitin-like domain. Among its many roles, ubiquitination targets proteins for degradation, and deubiquitination would be expected to disrupt this cellular pathway having effects on cell cycle control, genome integrity, and antigen presentation. The ubiquitin-like protein, ISG15, is strongly induced upon interferon treatment of cells and found conjugated to a few hundred cellular proteins. Although the role of this modification is unknown, it seems likely that reversing this modification by the action of the viral deISGylating activity could impair innate immunity and enhance viral survival and infectivity. Using a chromogenic substrate and the active site directed irreversible inhibitor, ISG15 vinylsulfone, we have shown that there are very few active deISGylating enzymes in mammalian cells. Uninduced cells exhibit deISGylating activity and the irreversible inhibitor labels both USP5/isopeptidase T and USP14. A cellular deISGylating enzyme, UBP43, is able to cleave these substrates and can be detected only in IFN-treated cells. In the case of USP5 and USP14 the cross reactivity appears to be due to the resemblence between ISG15 and diubiquitin. Usp5/isopeptidase T is very abundant but is not able to cleave ISG15-fusion proteins or the ISG15 conjugates that accumulate in IFN-treated cells. In contrast, the SARS papain like processing protease 2 (PLpro) readily cleaves fusion proteins and cellular ISG15-modified proteins that accumulate after IFN exposure. The results suggest that there are far fewer deISGylating enzymes than deubiquitinating enzymes in mammalian cells and that USP5 is not likely to be a significant factor in the response to IFN. Viral infections however, may well trigger both deubiquitination and deISGylation as part of the mechanisms of viral pathogenicity

The cellular ubiquitylation machinery is required for adenovirus entry

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Productive entry of adenoviruses in cells involves receptor mediated endocytosis and intra-endosomal partial disassembly of the viral capsid, followed by endosomal escape and transport to the nucleus. The process of endosomal trafficking and escape is at present ill defined. During partial intra-endosomal capsid disassembly, the structural protein VI is released from the inside of the virus capsid and recently it was demonstrated that protein VI lyses membranes in vitro. This lead to the hypothesis that protein VI is an adenoviral protein responsible for the penetration of the endosomal membrane.
Here we show additional data connecting protein VI to the cellular ubiquitylation machinery and the vesicular sorting system. We show that protein VI contains a functional ubiquitin ligase recruiting domain and that protein VI and penton are ubiquitylated following partial disassembly of the virus by a cytosolic activity. Depletion of the activity in vitro eliminates ubiquitylation of viral capsid proteins. We provide evidence for physical and functional association of protein VI with members of the Nedd4-family of HECT-E3-ubiquitin ligases and the cellular membrane system. Finally, RNAi knockdown of the E3 ligase inhibits adenovirus infection. We conclude that the vesicular trafficking and escape of adenoviral particles from the endosomal compartment is a highly regulated mechanism involving cellular, ubiquitin dependent, pathways.

Autophagosome supports coxsackievirus B3 replication in host cells

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Coxsackievirus B3 (CVB3), a small, non-enveloped, positive-stranded RNA virus, is an important human pathogen associated with myocarditis and dilated cardiomyopathy. It has recently been suggested that CVB3 utilize host machinery to enhance its efficiency of replication. However, the detailed mechanisms involved in viral replication are still unclear. Autophagy is a cellular process by which cells recycle cytoplasm and dispose of excess or damaged organelles. During this process, Intracellular targets are first sequestered by a double-membrane vesicle (autophagosome) and subsequently delivered to the lysosome for degradation. Autophagy plays an important role in developmental processes, human disease, and cellular response to nutrient deprivation. Defects in autophagy have been associated with tumor, neurodegenerative diseases, and cardiovascular diseases. Recent studies also suggest a critical role of autophagy in the control of viral replication. Autophagy has been primarily recognized as a host immune response to eliminate intracellular viruses. However, increasing evidence also suggests that autophagy may be utilized by certain viruses to enhance their efficiency of replication. In this study, we examined the potential role of autophagy in CVB3 infection.

To examine the potential role of autophagosome formation in CVB3 replication, HeLa cells were pre-treated with 3-methyladenine (3-MA), a pharmacological inhibitor of autophagy, or transfected with siRNAs to knockdown the genes (Beclin-1 and VPS34) critical for autophagosome formation. Cells were then infected with CVB3 at a MOI of 10. At 6.5h post-infection, the cell supernatants were collected for plaque assay analysis and the cells lyses were harvested for Western blotting. We found that inhibition of autophagosome formation either using 3-MA or by siRNAs significantly reduced viral protein expression and viral progeny titre. We also showed that treatment of HeLa cells with two known autophagy inducers, rapamycin and tamoxifen, prior to CVB3 infection resulted in an increased viral protein and progeny synthesis. To further determine the role autophagosome-lysosome fusion in viral replication, HeLa cells were pre-incubated with bafilomycin A and NH_4Cl, two pH-neutralizing agents known to inhibit lysosome protease function and prevent fusion of lysosome with autophagosome. We showed that treatment with both inhibitors promoted viral infectivity. The effect of blockage of fusion on viral infection was also confirmed by gene silencing of lysosome protein LAMP2. We showed that knockdown of LAMP2 enhanced viral replication.

Our results suggest that coxsackievirus utilizes host autophagic machinery for viral replication. Intracellular autophagosome may provide a physical scaffold to concentrate viral components and thereby increase viral replication.

E1B-55K has SUMO1-p53 ligase activity required for maximal p53 inhibition

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The adenovirus E1B-55K protein has been optimized by natural selection over a vast number of generations to rapidly inactivate p53, a key component in cellular anti-viral defense. E1B-55K employs several interrelated mechanisms to completely inactivate p53, culminating in p53 degradation. Here we report an additional E1B-55K function that contributes to p53 inactivation: Using highly purified recombinant proteins, E1B-55K stimulates the sumoylation of p53 in vitro, indicating that E1B-55K is a new class of SUMO1 E3 for p53; however, we are continuing experiments to rule out other possible explanations for the stimulation of p53 sumoylation. FRAP experiments in cells transfected with expression vectors for different PML isoforms indicate that the association of E1B-55K with p53 tethers p53 in promyelocytic leukemia (PML) nuclear bodies through interactions with PML isoform IV. p53-sumoylation in PML-nuclear bodies is mechanistically linked to p53 degradation by promoting rapid exportin1-mediated nuclear export of complexes containing p53 and E1B-55K followed by dynein motor-dependent transport on microtubules to the centrosomal microtubule organizing center (MTOC) for rapid ubiquitin-mediated proteolysis. Since viruses exploit normal cellular processes, these results predict that uninfected cells use similar mechanisms to inhibit nuclear proteins via sumoylation in PML-nuclear bodies followed by export to the MTOC and rapid proteosomal degradation.

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Wrestling with SUMO: The interaction between human adenovirus 5 E1A and the SUMO-1 conjugase UBC9

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Viral oncogenes have proven to be invaluable tools for probing the regulation of cellular processes. The E1A proteins are the immediate early gene products produced during an adenovirus infection. E1A functions include activating transcription of the other viral genes and promoting entry of the infected cell into S-phase, providing an optimum environment for viral DNA replication. E1A is an oncogene which can immortalize primary rodent cells or fully transform them in co-operation with a second oncogene, such as activated *Ras*. Numerous cellular proteins targeted by E1A have already been identified, including pRb, p300/CBP and CtBP. The effects of E1A on the functions of these and many other E1A interacting proteins have not been determined. *Saccharomyces cerevisiae* is a simple eukaryote that has been used extensively to understand the control of cellular processes. Certain strains of this yeast undergo filamentous growth/pseudohyphal differentiation (PD) when starved for nutrients. The changes in growth and morphology that accompany PD are easily visualized and the highly conserved cAMP and MAPK pathways that regulate PD are well characterized. We have identified a six amino-acid sequence in E1A (EVIDLT), spanning residues 118-123 in conserved region 2, which stimulates yeast pseudohyphal differentiation. This portion of E1A targets yeast Ubc9p and its mammalian equivalent UBC9 (a SUMO conjugase). Mutational analysis revealed that all residues except for E118 are required for this interaction. Interestingly, this same amino acid sequence is also present in a plethora of mammalian and yeast proteins that serve as SUMO E3 ligases. Thus we hypothesize that this sequence represents a novel motif that is present in at least some SUMO E3 ligases. The SUMO pathway has been implicated in many important cellular functions including cellular localization, transcriptional regulation, and cell cycle control. Interfering with the SUMOylation machinery has been observed when studying other viruses including avian adenoviruses. Currently, we are working to further elucidate the importance of this viral/host interaction with respect to its role in the viral life-cycle.

Ubiquitin ligase dependent budding of a lysine-free Gag protein

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Retroviral late or 'L-' domains, engage host ubiquitin ligases and/or class E VPS factors to mediate budding and multiple lines of evidence suggest that ubiquitin is important for their function. For example, PPXY L-domains recruit HECT ubiquitin ligases, whose catalytic activity is essential for particle release. Moreover, retroviral Gag proteins are often monoubiquitinated, and indications that direct Gag ubiquitination is critical arises from studies showing that mutation of multiple ubiquitin acceptor sites (lysine residues) in RSV and HIV-1 Gag proteins markedly inhibits budding.

A problem with studies involving multiple lysine mutations in Gag proteins is that assembly defects might be induced as a direct result of mutation rather than removal of ubiquitin acceptors. However, prototypic foamy virus (PFV) provides a potentially useful tool for assessing the requirement for Gag ubiquitination in budding because its Gag protein (remarkably) contains only a single lysine residue. Unfortunately, unlike conventional Gag proteins, PFV Gag requires co-expression of the viral Env protein to direct particle VLP assembly and release, and the Env cytoplasmic domain contains multiple ubiquitin acceptors that could usurp the function of ubiquitin acceptors in Gag.

Nevertheless, by insertion of a heterologous plasma membrane targeting signal at the PFV Gag N-terminus, we bypassed Env dependence for PFV Gag budding and generated a membrane-targeted lysine-free PFV Gag to test the role of Gag ubiquitination in L-domain function. PFV Gag encodes a PSAP late domain and, importantly, lysine-free Gag was efficiently released as VLPs in a PSAP and Tsg101 dependent manner. Remarkably, upon replacement of the PSAP motif in lysine-free Gag with the MLV PPXY L-domain motif, the MLV late domain retained its capacity to engage the HECT ubiqutin ligase WWP1 and supported particle release. Moreover, overexpression of catalytically active WWP1 markedly stimulated PPXY-dependent lysine-free Gag budding, while catalytically inactive WWP1 mutants did not. The activity of L-domains, and particularly the requirement for ubiquitin ligase activity in promoting lysine-free PFV (PPXY) Gag budding, strongly suggests that Gag ubiquitination is dispensable for L-domain function and further suggests the involvement of unidentified host ubiquitin ligase target(s) in virion release.

SUMO-2/3 modification and binding regulates CENP-E localization and progression through itomsis

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SUMO modification is essential for mitosis, but the protein targets and functions are poorly defined. Here, we demonstrate that distinct subsets of proteins are regulated by SUMO-1 and SUMO-2/3 modification during mitosis in mammalian cells. Blocking SUMO modification inhibited the localization of SUMO-2/3 modified proteins to centromeres and kinetochores of mitotic chromosomes without affecting the localization of SUMO-1 modified proteins to the mitotic spindle or spindle midzone. Notably, inhibition of SUMO modification caused a prometaphase arrest due to chromosome congression defects. A single defect consistent with the observed prometaphase arrest was detected; inhibition of SUMO modification prevented the targeting of CENP-E to kinetochores. In vitro and in vivo assays identified both SUMO-2/3 modification and SUMO-2/3 binding by CENP-E as essential regulators of its localization. Our findings define a novel paralogue-specific function for SUMO-2/3 modification and indicate that mitotic progression is controlled by SUMO-1 and SUMO-2/3 modification of distinct proteins.

Defining the Mechanisms of Paralogue Specific SUMO Conjugation: Analysis of the Bloom's syndrome DNA Helicase (BLM)

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The small ubiquitin-related modifiers (SUMOs) regulate diverse cellular processes through their covalent attachment to target proteins. There are three SUMO paralogues in mammalian cells: SUMO-1 shares approximately 45% identity with SUMO-2 and SUMO-3, which are 96% identical with each other and referred as SUMO-2/3. Different SUMO paralogues are believed to have distinct functions and to be conjugated, at least in part, to unique subsets of proteins in vivo. However, how different proteins are selectively modified by SUMO-1 or SUMO-2/3 is still unknown. Here, we show that BLM, a RecQ DNA helicase mutated in Bloom's syndrome, is preferentially modified by SUMO-2/3 relative to SUMO-1 both in vivo and in vitro. Yeast two-hybrid screening and in vitro binding assays indicated that BLM interacts non-covalently with SUMO-2/3 with a higher affinity relative to SUMO-1, leading us to hypothesize that non-covalent interactions between BLM and SUMO define its modification preference by facilitating substrate recognition. Consistent with this hypothesis we found that: (1) BLM has a SUMO-interacting motif (SIM) that mediates non-covalent interactions with SUMO and is critical for BLM modification, (2) excess SUMO proteins inhibit the modification of BLM, (3) BLM's SUMO-2/3 conjugation preference can be switched to SUMO-1 by swapping the SIM interaction surface in SUMO-2 with the same region in SUMO-1. Thus, our data indicate that noncovalent interaction between SUMO and BLM is required for sumoylation and that preferential paralogue modification is defined by the relative affinities of BLM for SUMO-1 and SUMO-2/3. We propose that non-covalent interactions between SUMO and target proteins may provide a general mechanism for mediating substrate recognition and paralogue specific modification.

Defining the Molecular Mechanism for SUMO Paralogue Specific Modification of RanGAP1

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SUMOs (Small Ubiquitin-related Modifiers) are covalently conjugated to a variety of proteins involved in different cellular processes. In mammalian cells, there are three SUMO paralogues, SUMO-1, SUMO-2 and SUMO-3. Based on their sequence similarities, the paralogues are divided into two subfamilies: a) SUMO-1 and b) SUMO-2 and SUMO-3. In vivo studies indicate that the SUMO paralogues are selectively conjugated, at least in part, to different subsets of proteins. However, the molecular mechanisms of paralogue specific conjugation are unknown. We have used RanGAP1 as a model substrate to investigate this issue. In vivo, RanGAP1 is specifically modified by SUMO-1, but it is equally well modified by both SUMO-1 and SUMO-2 in vitro. SUMO-1, but not SUMO-2 modified RanGAP1, however, was found to form a stable complex with Nup358 and was protected from deconjugation by SUMO isopeptidases in vitro. This finding provides a possible mechanism, based on protection from isopeptidases, for the apparent SUMO-1 specific modification of RanGAP1 seen in vivo. The specificity of Nup358 for SUMO-1 modified RanGAP1 is thought to be determined by a SUMO interaction motif (SIM) in Nup358 that binds more tightly to SUMO-1 than SUMO-2. To test the isopeptidase protection model, we replaced the Nup358 SIM interacting region in SUMO-1 with the same region from SUMO-2. The resulting SUMO-1 mutant was as efficiently conjugated to RanGAP1 in vitro as wild type SUMO-1, however, its binding to Nup358 was reduced. Consistent with reduced binding, RanGAP1/SUMO-1 mutant conjugates were less well protected from SUMO isopeptidases in vitro and failed to accumulate in vivo. Thus, our findings demonstrate that SUMO paralogue specific modification can be regulated at the level of deconjugation, by paralogue specific binding proteins that selectively protect SUMO-1 or SUMO-2 modified proteins from isopeptidases.

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